

**Expression studies and functional analysis  
of the genes *IDL4* and *IDL5*  
in *Arabidopsis thaliana***

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**ABSTRACT**

Cell-to-cell communication is a vital characteristic of multicellular organisms, making it possible for cells to coordinate their physiological behaviour. Small peptides may act as signalling molecules that control processes such as growth, differentiation and response to the environment. When such a ligand interacts with a receptor, it can trigger downstream effects like intracellular responses or change of gene expression. Recently, a novel group of putative peptide ligands, the IDA-LIKE (IDL) proteins was discovered, based on their similarity to IDA. IDA is a putative ligand involved in floral abscission (Butenko *et al.* 2003). For this thesis, the *IDA-LIKE* genes *AtIDL4* and *AtIDL5* in the model plant *Arabidopsis thaliana* have been studied.

Histochemical analysis of promoter-GUS constructs for *IDL4* and *IDL5* have been performed in transgenic *Arabidopsis* plants. Promoter activity showed gene function in a wide range of tissues to be probable. Noticeably the *IDL4* promoter was activated in tissues throughout the life span of the plant, whereas *IDL5* activity was shown exclusively in young parts.

An insertional mutant line with an immobilised transposable element situated in the *IDL5* coding region was analysed. No striking phenotype was observed, but microarray expression profiling was undertaken in search for molecular phenotypes.

Over expression of the two genes led to similar aberrant phenotypes. Plants were generally smaller, and had curled leaves that appeared water-soaked. They also showed increased guttation and white, crystalline patches often appeared on leaf margins. Some transformants developed abnormal siliques and displayed reduced fertility. A minority of the individual plants over expressing *IDL5* developed enlarged floral abscission zones and cells there were covered in a white substance.

One could tentatively suggest that the *IDL* genes show activity at sites where cell separation processes occur, however expression was not restricted to such regions and this hypothesis would require further assessment. *IDL4* and *IDL5* may also be involved in the formation and/or function of hydathodes. Both genes' promoter showed activity in these pores, and the gain-of-function mutants displayed increased guttation. Additional analyses will be required to further understand the functions of the *IDL* genes.



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## 1. INTRODUCTION

### 1.1 *Arabidopsis thaliana* as a model organism

*Arabidopsis thaliana* is a small flowering plant in the *Brassicaceae* (mustard) family. It was reported used as a model organism for plant biology nearly a century ago. However, not until the mid 1980ies and the advent of powerful molecular biology methods this particular species became recognised as a useful model system (Somerville and Koornneef 2002). Some of the advantages appreciated by researchers are the plant's small size and simple growth requirements, short life cycle (6-8 weeks) and the production of thousands of seeds from each individual. *Arabidopsis* is a self-pollinating plant, so that cross-pollination can be controlled. The additional discovery that *Arabidopsis* plants can easily be transformed with foreign DNA using a system based on the soil bacterium *Agrobacterium tumefaciens* made *Arabidopsis* the natural choice for plant molecular biologists (Meinke *et al.* 1998).

Following the growth of the *Arabidopsis* research community, several resources have been established. The full sequence of the plant's 125 Mb genome was released in 2000 (*Arabidopsis* Genome Initiative 2000), and there are large collections of characterised mutants and sequenced transgenic insertion lines available. This facilitates the discovery and analysis of new genes and gene families. It is indeed a stated goal within the *Arabidopsis* community to understand the function of all *Arabidopsis*' ~25 500 genes by the year 2010 (Chory *et al.* 2000).

### 1.2 Generation of transgenic lines

#### 1.2.1 *Agrobacterium tumefaciens*

*Agrobacterium tumefaciens* is a soil-dwelling, gram-negative bacterium. It has the remarkable ability to transfer parts of its own DNA to plants. In nature, *Agrobacterium* manipulates plants to form tumors of cells that produce and release a group of compounds called opines. Few other microorganisms can catabolise these substances, thus by successfully attacking plants, *Agrobacterium* forms a favourable biological niche for itself (Zupan *et al.* 2000).

The virulence genes (*Vir*), which are involved in processing, transport and integration of the transferred DNA (T-DNA), are situated on the Ti plasmid. The Ti plasmid also contains the T-DNA itself, flanked by two 25 bp imperfect repeats, denominated border sequences. The T-DNA borders on the Ti plasmid are recognised and cleaved by the proteins VirD1 and VirD2 (Filichkin and Gelvin 1993). VirD2 becomes covalently attached to the 5' end of the single

stranded T-DNA. The T-DNA/ VirD2 complex, together with VirE2 protein, is delivered to the plant cytoplasm through the VirB pili. Once inside the plant cell, the single stranded T-DNA becomes coated with VirE2 proteins, which protect the T-DNA from being degraded by plant nucleases (Rossi et al. 1996). Both the VirD2 and VirE2 proteins include sequences that are recognised as nuclear localization signals in plants, and the T-DNA complex is imported to the nucleus through the plant's own importins (Ballas and Citovsky 1997).

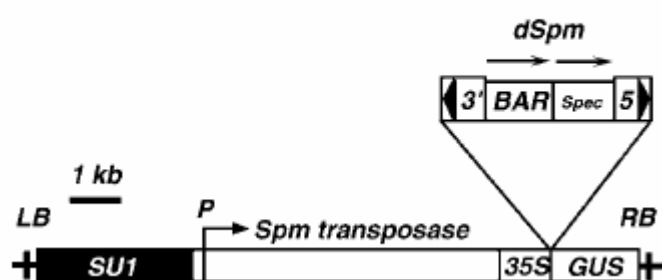
The bacterial DNA is incorporated into the plant genome by illegitimate recombination. This is a process where two DNA molecules are joined without extensive homology. A model for this integration process has been proposed (Tinland and Hohn 1995; Brunaud *et al.* 2002), but little is known about which proteins are involved (Ziemienowicz 2001). The integration is thought to rely upon micro similarities between the host DNA and the T-DNA Left Border (LB) region. Because only a low level of similarity is required for T-DNA insertion, it can virtually integrate anywhere in the plant genome.

### **1.2.2 The transformation of *Arabidopsis* plants**

Transformation of *Arabidopsis* plants is routinely performed by using the bacterium *A. tumefaciens*. The two 25 bp T-DNA borders are the only sequences of the T-DNA that are necessary for the introduction into plant genomes. Any DNA sequence introduced between the left and right borders will be transferred to the plant genome, resulting in the integration of one or more T-DNAs into the nuclear genome at one or several independent loci (Koncz et al. 1989). A common transformation protocol for *Arabidopsis* is the floral-dip method where the flowers are dipped in a solution with bacteria, which will lead the bacteria to transform the germ line cells that make the female gametes (Bechtold et al. 2000). The *A. tumefaciens* transformation system has been successfully used to create loss-of-function or knockdown mutants by the random insertion of T-DNA sequences into coding or regulatory sequences of genes. Other applications include promoter trapping, expression studies, activation tagging and genetic complementation. A large collection of T-DNA insertions (Alonso et al. 2003) have been sequenced and made available to the public (<http://signal.salk.edu/cgi-bin/tdnaexpress>). Here, one can search for insertions in one's gene of interest, and order seeds from stock centres.

### 1.2.3 Transposable elements

Transposable elements, or transposons, are genetic fragments that are capable of moving within genomes. The concept of such fragments was developed by Barbara McClintock in the 1950ies, and has been widely studied in *Zea mays* (Gierl and Saedler 1989). By changing their chromosomal location, transposons can create insertional mutations and disrupt the function of genes. Transposable elements generally require one or more enzymes (transposases) that facilitate the excision and reinsertion, and sequences that serve as substrates for the transition. If all the required elements are present on the transposon itself, it is said to be autonomous. If it needs the enzymes activated in trans (i.e. internal deletion derivatives), it is called a defective element (Gierl and Saedler 1989). The *En/Spm* transposable element has been widely used for insertional mutagenesis and isolation of disrupted genes (transposon tagging) in *Zea mays*, and has been shown to function in *Arabidopsis* as well (Cardon GH 1993). The autonomous *En/Spm* transposon encodes the transition element *I/dSpm* and the two transposases *tnpA* and *tnpD*. Several deletion derivatives exist where the transposases are inactive and hence the substrate sequence is immobile (Cardon GH 1993). Such defective elements can be activated by autonomous transposons elsewhere in the genome. The SLAT (Sainsbury Laboratory *Arabidopsis thaliana* Transposants) collection, created by the John Innes Centre (Tissier et al. 1999) provides insertion mutants created by the use of transposable dSpm elements. When constructing the library, plants were transformed with the autonomous Spm transposon, i.e. with the transposable element and the gene for its transposase in the same T-DNA (fig 1.1). The T-DNA with the transposase sequence harboured a counter-selectable marker and was crossed out after the transposition, preventing further movement of the inserted sequence. More than 24 000 independent lines have had their insertion site sequenced, and interesting lines can be found at databases such as <http://signal.salk.edu/cgi-bin/tdnaexpress>



**Figure 1.1 The T-DNA construct used to generate the SLAT library.**

LB and RB, left and right borders, respectively; P, promoter driving the expression of the transposase; BAR, phosphinothricin resistance gene; Spec, spectinomycin resistance gene for selection in bacteria; SU1, counterselectable marker that activates a proherbicide. (Tissier et al. 1999)

### 1.3 RNA interference

RNA interference is a process in eukaryotes that can be used to reduce expression of specific genes. The system involves cleavage of double stranded RNA (dsRNA), and probably originated as a defence system against viruses (Baulcombe 2004). Modern plants have additionally evolved to use similar mechanisms for gene regulation and to shield themselves from the effects of (retro-) transposons.

dsRNA in plant cells are cleaved by a ribonuclease III enzyme (dicer) into 21-26 nucleotide RNAs, referred to as short interfering RNAs (siRNA). The dsRNA processing by dicer is most likely to occur in the nucleus (Papp et al. 2003) and siRNA is transported to the cytoplasm, where it associates with the RNAi silencing complex (RISC). RISC has nuclease activity, and degrades mRNAs that are complementary to the now single stranded siRNA in the complex. In addition dsRNA can also induce processes that lead to sequence-specific DNA methylation. Methylation of DNA can cause changes in the local chromatin structure, thereby silencing genes by blocking their transcription (Mette et al. 2000).

In functional analysis, the RNAi silencing pathway is triggered by sequence-specific hairpin transgenes, and gene function can be elucidated based on any aberrant phenotypes in the transformants. Insertional mutagenesis has several obstacles that RNAi can help overcome. Firstly the *Arabidopsis* genome is far from saturated with knockout insertions. The probability of finding an insertion in a given gene is given by the equation

$$P = 1 - [ 1 - ( X / 125\,000 ) ]^n$$

where X is the size of the gene in kilo bases, and n is the number of insertions available (Østergaard and Yanofsky 2004). Thus, if one is working with a gene smaller than 1 kb, there is less than a 60% probability of finding an insertion in that gene if the database contains 100 000 insertion lines. Conversely, RNAi can target specific genes and makes the goal of dissecting the function of every *Arabidopsis* gene more feasible. Secondly, complete silencing of genes required for basic cell or developmental could lead to embryo lethality in some cases. Such mutations would not be easily recovered and further studied. Two features of RNAi help work around this particular challenge; RNAi often produce transformants with a wide degree of partial silencing (Wesley *et al.* 2001) and it can be made inducible (Guo et al. 2003). However, a weakness with silencing genes post-transcriptionally is that its effectiveness depends on the turnover rate of the mRNA in question (Fire 1999). mRNA that is rapidly synthesised and degraded might therefore be less affected by RNAi silencing.

## 1.4 Functional studies

### 1.4.1 The Promoter-GUS assay

In the promoter::GUS assay, plants are transformed with a construct harbouring the expected promoter cloned in front of a reporter gene,  *$\beta$ -glucuronidase (GUS)*. If the promoter is activated, *GUS* will be transcribed and any added X-gluc (5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide) substrate will be hydrolysed into a water-soluble indoxyl intermediate. The product is further dimerised into a dichlorodibromo blue precipitate by an oxidative reaction. A blue colour in the histochemical assay will thus reveal where and when the gene in question would normally be expressed.

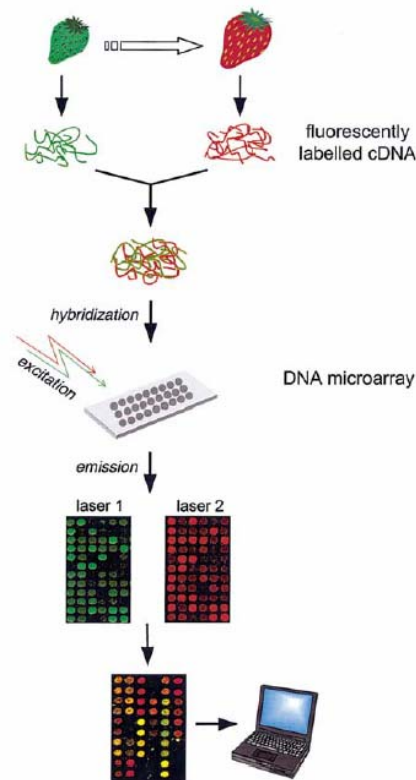
*GUS* is to date the most commonly used reporter gene in transgenic plants (Gilissen et al. 1998). The gene is derived from *E. coli*, and catalyses the hydrolysis of a variety of glucuronides. GUS is a robust enzyme and is not harmful to plant hosts (Jefferson et al. 1987). Higher plants have no intrinsic GUS activity, thus small amounts of GUS activity will not be masked by background noise (Jefferson 1989). The GUS enzyme's substrates include the sensitive histochemical (X-gluc) and several chromogenic and fluorogenic substrates for more quantitative assays.

### 1.4.2 Microarray analysis

DNA microarray is a powerful tool for studying full-genome expression. The technology is a hybridisation-based method where the relative mRNA levels between two samples can be compared. Nucleotides representing unique mRNAs are immobilised to a solid surface such as glass slides. There are currently two strategies utilised in manufacturing DNA microarrays: cDNA microarrays, which involves deposition of DNA fragments such as cDNA clones to slides, and oligonucleotide microarrays. The latter may be manufactured by *in situ* synthesis of oligonucleotides on the solid surface and require more extensive knowledge of the genome sequence (Wu et al. 2001).

The principle of the technology involves reverse-transcription of mRNA from two samples, where the resulting cDNA is labelled with different fluorescent dyes (e.g. with the presence of Cy3/ Cy5-dUTP). The differently labelled cDNA are subsequently mixed and hybridised to the same microarray slide. The slide has spots with oligonucleotides representing individual transcripts. After hybridisation, the slide is scanned with a confocal laser at the two appropriate wavelengths. The fluorescent intensities in the array spots should correspond to

the amount of specific mRNA in each sample. The measured abundances are obtained on a relative rather than an absolute scale. This is because they depend on factors such as the efficiencies of the various chemical reactions involved in the sample preparation, as well as on the amount of immobilised DNA available for hybridisation (Huber et al. 2003). Figure 1.2 shows a scheme of a typical microarray experiment comparing gene expression at different developmental stages. The same principles could be used to compare tumors to healthy tissue, mutant vs. wildtype, tissues with or without stress etc.



**Figure 1.2 Scheme of a typical cDNA microarray assay for gene expression analysis.**

mRNA levels are compared between the green and red stages of fruit development. First, mRNA is isolated from each tissue and reverse-transcribed in the presence of different fluorescent dyes resulting in labelled cDNA. Next, the two cDNA populations are mixed and hybridised to a microarray with probes that contains DNA representing a specific gene. The cDNAs from both populations will hybridise specifically with the probe on the corresponding array element. After hybridisation, the microarray is scanned and the relative abundance of mRNA from each gene in green vs. red fruit is reflected by the ratio green/red as measured by the fluorescence emitted from the corresponding array element. (Aharoni A 2002)

After intensity values have been obtained by image software, careful analysis of the data is required. A single microarray slide may contain the equivalent to tens of thousands of single hybridisation experiments. The resulting data could contain experimental variation from a variety of sources (Huber et al. 2003), which need to be accounted for mathematically. Several algorithmic tools for calibration and normalization have been developed (Smyth and Speed 2003).



## 1.5 Aspects of *Arabidopsis* development and defence mechanisms

### 1.5.1 Hydathodes and guttation

Secretion is an essential process for plants to dispose of unwanted solutes, improve nutrient uptake and attract other organisms (Pilot et al. 2004). Excretion in leaves is mediated by specialised structures called hydathodes, pores formed in the leaf margins close to minor vein endings. The process whereby hydathodes secrete a sap containing ions, metabolites and proteins is termed guttation (Komarnytsky et al. 2000). One hypothesis is that hydathodes retrieve organic molecules and ions from the apoplast and exude water - much like kidneys in animals. Data supporting this hypothesis include expression of several transporters in hydathodes such as for potassium, sulphate and N-heterocycles (Lagarde *et al.* 1996; Burkle *et al.* 2003). Secretion could alternatively be driven by active export of osmolytes and passive efflux of water (Pilot et al. 2004). Little is currently known about the development of hydathodes or the exact mechanisms behind guttation, however the formation of the pores probably involves cell separation. Increased expression of the cell wall modifying enzyme polygalacturonase (PG) has been reported in developing hydathodes (Roberts et al. 2002).

As of yet, no loss-of-function mutant affecting hydathode function has been described for *Arabidopsis*. An activation-tagging screen revealed that over-expression of the gene *GLUTAMINE DUMPER1* (*GDUI*), encoding a previously undescribed transmembrane protein, led to increased guttation and formation of salt crystals on the leaf margins. *GDUI* was suggested to encode an amino acid transporter (Pilot et al. 2004).

Hydathodes are constitutively open pores, and may serve as an entry point to many micro-organisms, including pathogens (Hugouvieux et al. 1998). Analysis of the guttation fluid in barley (*Hordeum vulgare*), showed that most of the approximately 200 different proteins found there could be assigned to pathogenesis-related (PR) defence proteins (Grunwald et al. 2003).

### 1.5.2 *Arabidopsis*' response to mechanical damage and pathogen attack

Sessile plants are often exposed to mechanical damage from factors such as wind, rain, hail and herbivore feeding. Wounding is a threat to plant survival, both because of the destruction of plant tissues, and because it presents an entry-point for invading pathogens. Therefore, it is hypothesised that plants have evolved defence pathways that integrate the response against mechanical wounding and pathogens (Cheong et al. 2002).

The plant encodes a number of proteins involved in different defence mechanisms. These proteins include pathogenesis-related proteins (PR), phytoalexins (toxins targeting pathogens), enzymes for oxidative stress protection, tissue repair and lignification (Reymond and Farmer 1998). Many of the genes encoding these proteins are regulated by the plant hormones jasmonic acid (JA), salicylic acid (SA) and ethylene. Results point towards the notion that other phytohormones and small signalling compounds may additionally be involved in regulating defence responses (Reymond and Farmer 1998).

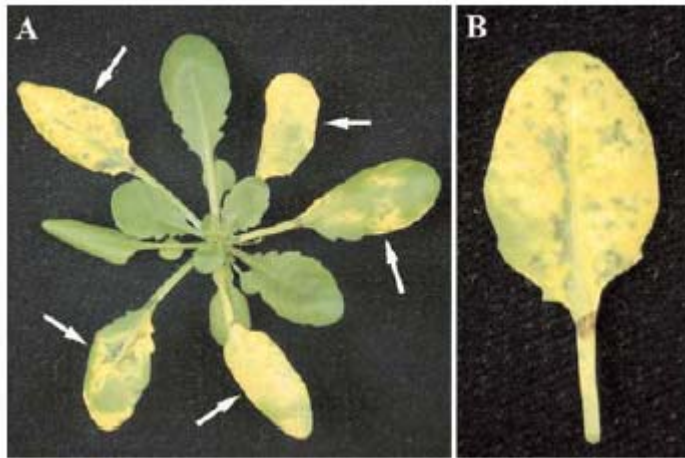
#### 1.5.2.1 The *P. syringae*–*A. thaliana* interaction as a pathogen–host model system

*Pseudomonas syringae* is a gram-negative, rod-shaped bacterium with polar flagella that infect a variety of plants. Strains of *P. syringae* show diverse and host-specific interactions with plants, and are named after the pathovars (pv) in which they originally were collected. This bacterium was the first pathogen discovered to infect *Arabidopsis* and cause disease in laboratory settings. To date, no reports have been made of *P. syringae* causing disease on *Arabidopsis* in the wild, and the infection in laboratories require the use of surfactant or pressure infiltration. Nevertheless, the bacterium is now a widely accepted system for genetic analysis of plant-pathogen interaction (Katagiri et al. 2002).

*P. syringae* enters the host tissues, usually leaves, through wounds or natural openings such as stomata and multiplies in the intercellular space in susceptible plants. Water and perhaps nutrients leak from infected *Arabidopsis* leaf parts, and after some time the patches become necrotic (Fig 1.3). The most commonly used strains of *P. syringae* in *Arabidopsis* research are *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola* ES4326.

Some strains of *P. syringae* encode genes which products are recognised by certain plant host. These pathogen genes are termed *avirulence* (*avr*) genes, and the host genes responsible for the recognition are dubbed *resistance* (*R*) genes. Plants with a certain R gene do not show signs of disease upon infection with bacteria expressing the corresponding *avr* gene (Flor 1971). When the plant is resistant, the pathogen is said to be avirulent and the interaction is said to be incompatible. When the plant is susceptible, the pathogen is said to be virulent and the interaction is said to be compatible (Katagiri et al. 2002). *Arabidopsis* has been important in elucidating the molecular basis for this important gene-to-gene pathogen interaction in

plants. *Arabidopsis* also shows a general resistance response, where even compatible interactions are slowed down, although not effectively enough to prevent disease.



**Fig 1.3 *P. syringae* infection**  
Disease symptoms in *Arabidopsis* leaves caused by DC3000 infection. Leaves (indicated with arrows) were syringe-infiltrated with  $5 \times 10^5$  cfu/mL of *Pst* DC3000 and pictures were taken four days after inoculation. The whole plant is shown in (A). A close-up of a diseased leaf is shown in (B). (Katagiri et al. 2002)

#### 1.5.2.2 *Botrytis cinerea* and *Arabidopsis*

*Botrytis cinerea* is a fungal pathogen causing great loss to fruit and vegetable crops (Tournas 2005). In contrast to biotrophic pathogens, such as many bacteria, *B. cinerea* is a necrotrophic microorganism that actively kills host tissue to obtain nutrients. It is widely believed that plants elicit different defence responses toward pathogens of this kind (Denby et al. 2004). The hypersensitive response (HR), which is a part of the classic avr-R pathway protecting the plant from several biotrophic pathogens, has even been shown to aid the *B. cinerea* in colonizing the plant (Govrin and Levine 2000). The *B. cinerea*-*Arabidopsis* interaction is often used as a model for molecular studies of plants' responses to necrotrophic pathogens, e.g. (Govrin and Levine 2000; Denby et al. 2004; Kishimoto et al. 2005).

### **1.6 Cell-cell signalling in plants**

A vital function in multicellular organisms is communication between cells, which is necessary to control and orchestrate differentiation, growth and responses to environmental stimuli. Until the beginning of the 1990ies, most cell-to-cell communication in plants were described with the "classical" plant hormones auxin, cytokinin, ethylene, gibberilin-and absisic acid (Kende and Zeevaart 1997), as well as other non-peptide hormones (Creelman and Mullet 1997). Recently, discoveries have indicated that plant cells, like those of animal origin, also use small peptide signals and specific receptors to communicate (Matsubayashi 2003).

### **1.6.1 Peptide ligands in plants**

Systemin in tomato was the first polypeptide signal to be discovered in plants (Pearce et al. 1991). It is an 18-residue polypeptide derived from a 200-aa precursor that is released upon wounding during herbivore or pathogen attack. Since 1991, a few other groups of plant signal peptides and their receptors have been reported. Phytosulfokines (PSK) are 5-residue peptides with sulphated tyrosines that regulates cell proliferation and differentiation (Matsubayashi et al. 2002), CLAVATA3 is a 73-aa polypeptide which has been shown to regulate meristem cell fate in *Arabidopsis* (Fletcher et al. 1999) and 55-to 58-aa cystine-rich polypeptides (SCRs) regulate self-incompatibility in *Brassica*. The IDA-like proteins (Butenko et al. 2003), the DEVIL family (Wen et al. 2004) and the RTFL (Rotundifolia Four Like) peptides (Narita et al. 2004) are recently discovered signal peptides in *Arabidopsis* where little or no evidence for specific receptors exist yet.

Signal peptides are often encoded by small genes, and therefore commonly overlooked by automated annotation programs (Ride et al. 1999). A common feature in some groups of putative peptide ligands is the presence of an N-terminal signal sequence that targets the transcribed protein through the secretory pathway to the extra cellular space and is proteolytically cleaved.

### **1.6.2 Receptor-like kinases in plants**

Based on sequence homology, more than 600 genes encoding RLKs are found in the *Arabidopsis* genome, making it one of the largest gene families in the species (Shiu and Bleecker 2001b). They are characterised by having an N-terminal transmembrane domain, a signal sequence and a C-terminal ser/thr kinase domain (Walker 1994). The gene family is further divided into 44 subfamilies, based on the identity and organization of their domains (Shiu and Bleecker 2001a). Some of these subfamilies of receptor protein kinases lack the transmembrane domain, and are referred to as receptor-like cytoplasmic kinases (RLCKs).

RLKs vary greatly in their extra cellular domain, which have been shown to bind substances such as brassinosteroids, peptides, glycoproteins and microbial cell wall components (Shiu and Bleecker 2001a). 235 of the RLKs in *Arabidopsis* have an extra cellular domain with 1-32 leucin-rich repeats (LRRs) that often participate in protein-protein interactions (Kobe and Deisenhofer 1994). Members of the LRR-RLK subclass have been found to regulate various developmental processes, phytohormone perception and defence responses. The

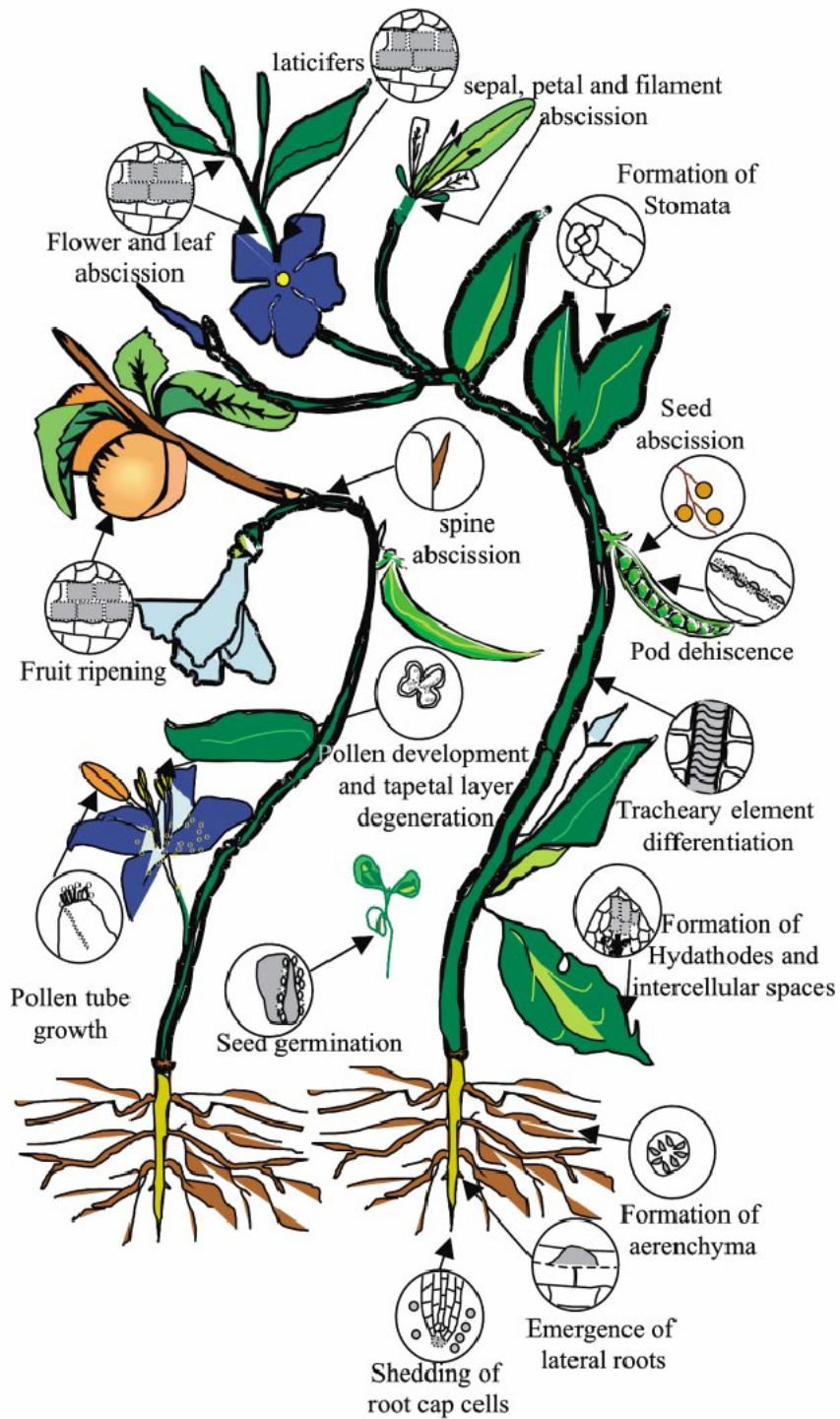
developmental regulators in *Arabidopsis* include the proteins ERECTA (organ shape), CLAVATA1 (meristem cell fate) and HAESA (floral abscission) (Torii 2000).

On the whole, RLKs with known function represent only a fraction of the total number of putative RLKs identified. Even less is known about their interactants and ligands.

### **1.7 Cell separation processes**

When plant cells divide to proliferate, the two daughter cells are normally joined together by a cellulose wall that provide strength, but also restrict the activity and autonomy of newly formed cells. In a number of structures it is crucial to circumvent these restriction, and controlled processes to loosen or break adhesive bonds occur during development or as a response to environmental clues (Roberts *et al.* 2000; Roberts *et al.* 2002). Examples of important cell separation processes in plants include the development of intercellular air spaces in expanding leaves, fruit ripening, releasing of pollen, organ separation (abscission), pod shatter, formation of transmitting tract in the style, lateral root emergence and root cap cell detachment (Hong *et al.* 2000). Figure 1.4 highlights parts of the plant where such processes are likely to take place.

Concomitant with cell separation is the increased expression of hydrolytic enzymes that facilitates cell wall degradation, such as cellulase, polygalacturonases and expansins (Roberts *et al.* 2002). Cell separation may provide a point of entry for invading pathogens. Unsurprisingly, several pathogenesis-related (PR) defence proteins accumulate at the site of separation (Roberts *et al.* 2002). Both abscission and dehiscence are specialised cell separation processes that occur at discrete sites with predetermined cells (Roberts *et al.* 2000). Abscission refers to a separation event where whole organs, such as leaves, seeds or flowers are shed. Dehiscence, on the other hand, results in the opening of a plant organ such as the silique.



**Fig 1.4 Sites of cell separation in plants**  
(Roberts *et al.* 2002)

### 1.7.1 Abscission

Abscission is a process where plants shed organs in a controlled manner. The process provides a mechanism to release fruit, remove organs that are damaged or have served their purpose (Bleecker and Patterson 1997). Abscission takes place at morphologically distinct bands of small cells with dense cytoplasm, termed abscission zones (AZs) (Sexton and Roberts 1982). These zones are usually defined early in development at the base of organs that are to be shed (Gonzalez-Carranza et al. 1998). During the abscission process the middle lamella between the AZ cells dissolves, followed by a cell enlargement. The cells on the proximal face of the resulting fracture plane differentiate into protective scar tissue (Addicott 1982).

The balance between the two plant hormones ethylene and auxin has long been known for its role in timing abscission (Sexton and Roberts 1982); ethylene promotes organ shedding and auxin delays it (Gonzalez-Carranza et al. 1998). However, studies of plants with disrupted ethylene sensing, such as plants with decreased levels of the putative ethylene receptor ETR-1, have shown that ethylene is not an absolute requirement for abscission to take place.

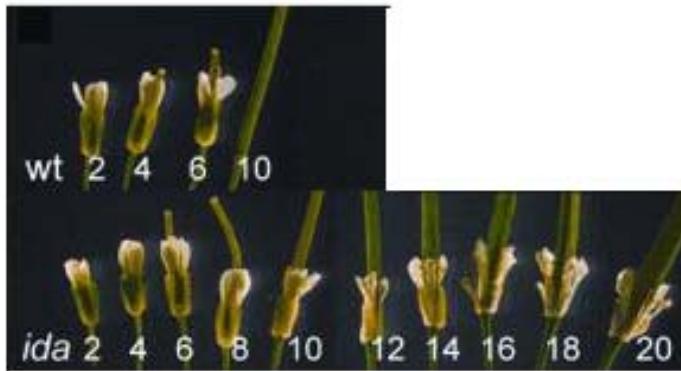
*Arabidopsis* displays abscission of mature seeds (Pinyopich et al. 2003) and floral organs (Patterson 2001). The seed is connected to the fruit through an umbilical cord-like structure, the funicle. Seeds are released through an abscission process and the seed abscission zone (SAZ), which is situated immediately adjacent to the seed body, is differentiated after fertilization (Pinyopich et al. 2003). Few studies have been undertaken to unravel the mechanisms behind seed abscission (Jenkins et al. 1999), but the gene *SEEDSTICK* (*STK*), encoding a MADS-box transcription factor involved in ovule development, was shown to control both funicle development and seed abscission (Pinyopich et al. 2003). Genes involved in floral abscission are starting to be revealed by mutant and antisense studies. *HAESA* encodes a LRR-RLK and shows expression in floral abscission zones (FAZ) and anti-sense *HAESA* lines show delayed floral abscission (Jinn et al. 2000).

### 1.8 INFLORESCENCE DEFICIENT IN ABSCISSION (*IDA*) and the *IDA-LIKE* genes

*Arabidopsis* sheds its floral organs shortly after anthesis in a controlled abscission process (Bleecker and Patterson 1997). Butenko and colleagues discovered a mutant that despite it having differentiated AZ cells its floral organs remain attached throughout its life cycle (Fig 1.5) (Butenko et al. 2003). A complementation assay showed that the mutation was caused by



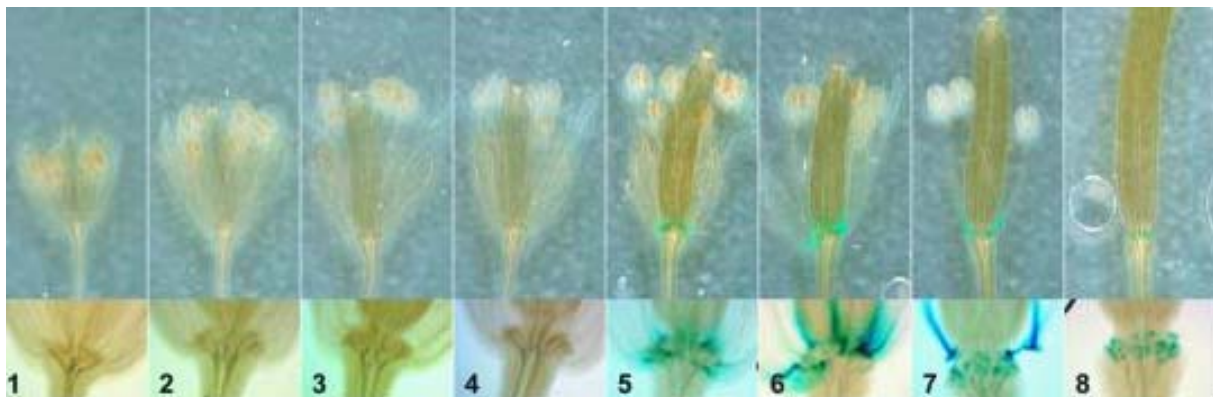
an insertion in the promoter region of the gene At1g68765, later referred to as *IDA*, *INFLORESCENCE DEFICIENT IN ABSCISSION*. *ida* shows normal ethylene sensitivity; hence the deficiency of abscission is not affected by ethylene



**Fig 1.5 The *ida* phenotype**

Numbers indicate position from the first flower with visible white petals at the top of the inflorescence. The uppermost part displays abscission of wildtype C24 floral organs shortly after anthesis. *ida* floral organs remain attached indefinitely. (Butenko et al. 2003)

The expression of *IDA* has been investigated with a promoter::*GUS* assay. *GUS* expression was confined to the base of all floral organs (anther filaments, petals and sepals) and their AZ, as well as the outgrowths of nectaries. Prior to abscission (stages 1-4 of floral development), *GUS* expression was absent. *GUS* activity was strongest in the AZs through the floral stages 5-9, concurrent with the abscission process. At later stages, *GUS* expression was seen solely at the nectary outgrowths (Fig 1.6). (Butenko et al. 2003)



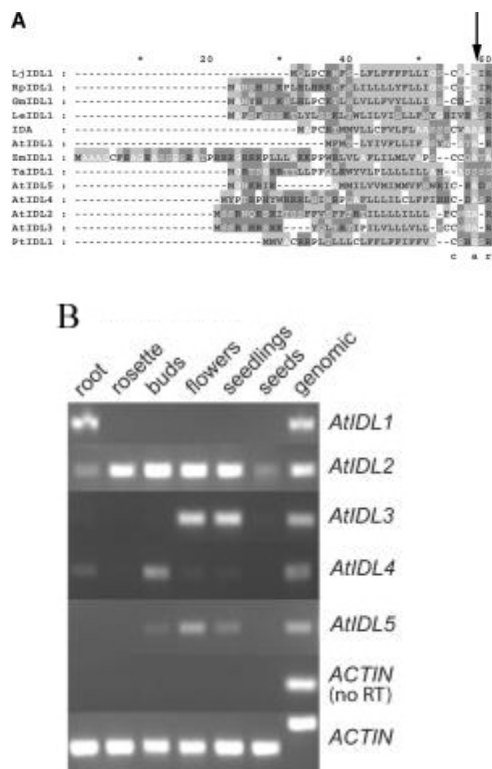
**Fig 1.6 *IDA*- promoter::*GUS* expression**

Developmental assay showing stage-specific AZ *GUS* expression in early *IDA*::*GUS* flower stages. Top row, whole-flower overview; bottom row, AZ detail. Arabic numerals indicate flower positions on the inflorescence. Note the absence of *GUS* activity before the onset of the abscission process. At position 5, *GUS* was detected. *GUS* was expressed throughout the abscission process in both AZ and at the base of abscised organs. (Butenko et al. 2003)



The *IDA* gene encodes a small protein of 77 amino acids with a high pI (11.87) and an N-terminal hydrophobic region predicted to act as a secretory signal peptide (Butenko et al. 2003). Onion epidermis cells transiently transformed with an *IDA::GFP* fusion construct, showed that both *IDA* and the signal peptide alone localise to the extra-cellular space. The signal peptide, high pI and small size are similar to putative classes of secreted peptide ligands like the CLAVAT3-like (CLE) (Cock and McCormick 2001) and SCR-like proteins (Vanoosthuyse et al. 2001). However, the C-terminus of *IDA* is distinct from the motifs that are characteristic to other known classes of putative ligands.

A tBLASTn search with the C-terminal 20 residues of the *IDA* protein against plant EST collections and the translated *Arabidopsis* genome identified several *IDA-like* (*IDL*) transcripts. They all share similar characteristics in having predicted N-terminal signal sequences, similar pI values (ranging from 11.02 to 12.62), and a conserved C-terminal signature (pv/iPpSa/gPSk/rk/rHN), which was termed PIP (Butenko et al. 2003). Putative *IDL* genes were found in transcripts from eight different plant species, including one in *Arabidopsis* (*AtIDL1*). Four novel genes (*AtIDL2-5*) were found in the *Arabidopsis* genome (Fig 1.7 A). *AtIDL1-5* were shown to be expressed in diverse tissues with RT-PCR (Fig 1.8 B). The diverse expression patterns differ from that of *IDA*, and led to the conclusion that the *IDL* genes may be involved in diverse processes in the plant (Butenko et al. 2003).



**Fig 1.7 The IDL genes**

(A) Alignment of *IDA* and *IDL* proteins encoded by cDNAs from *Arabidopsis* (*AtIDL1-5*), tomato (*LeIDL1*), lotus (*LjIDL1*), soybean (*GmIDL1*), black locust (*RpIDL1*), maize (*ZmIDL1*), poplar (*PtIDL1*) and wheat (*TaIDL1*). Note the arrow indicating the positions of cleavage sites predicted by SignalP.

(B) RT-PCR shows that the *Arabidopsis* *IDL* genes are expressed in various tissues.

(Butenko et al. 2003)

### **1.9 Aim of this study**

This study is part of a larger project, which goal is to characterise the members of the *IDL* putative gene family. The aim of this thesis was to further investigate *AtIDL4* (At3g18715) and *AtIDL5* (At1g76952), by using expression studies and functional analysis. Promoter::*GUS* analysis was used to characterise the expression pattern of the two genes, both during normal development and biotic stress. To further understand the biological function of the genes, a knockout insertion line for *AtIDL5* was investigated and an RNAi knockdown line was generated for *AtIDL4*. Microarray analysis was used to search for molecular phenotypes in the *idl5* insertion mutant. Over-expression lines were generated for both genes

## 2 MATERIALS AND METHODS

### 2.1 Plant methods

#### 2.1.1 Seed sterilisation

Seeds (50-200) were added to a 1.5ml eppendorf tube. Working in sterile conditions, the seeds were washed with 70% ethanol for 5 min. The ethanol was removed and bleach solution (20% clorix, 0.01% Tween20) was added and incubated for 5 min. The solution was removed, and the seeds were washed with 0.0001% Tween20, followed by washing with sterile water. The seeds were resuspended in 1.5 ml 0.1% Difco agar and spread on a MS2 plate (4.43 g/L Murashige and Skoog, 0.5g/L 2-N-morpholinoethanesulphonic acid (MES), 20.0 g/L sucrose, 5g/L Difco agar, pH= 5.7) (Murashige T 1962). Kanamycin (Km) 50µg/mL was used for selection of plants containing Km resistance. At the University of Cape Town (UCT) a different method for sterilization of seeds was used. Seeds were shaken for 7 minutes in 70% ethanol, before incubation in 10% bleach and 0.02% Triton-X100 for 15 minutes. The sterilised seeds were rinsed 5 times in mQ distilled water. Here, seeds were incubated in 0.1% agar in the dark at 4°C for at least 4 days before plating.

#### 2.1.2 Growth conditions

Sterilised seeds on MS2-plates (Murashige T 1962) were incubated in the dark for 3-4 days at 4°C before transferred to growth room. The growth room conditions were 22°C, 8 h dark and 16 h light (100 µE m<sup>-2</sup> sec<sup>-1</sup> of intensity.) After 3-4 weeks plants were placed on soil under the same growth conditions. At UCT seed were plated on Plant Nutrient (PN) agar plates without sucrose as described in (Haughn 1986). Seedlings were grown in continuous light (100 µE m<sup>-2</sup> sec<sup>-1</sup>), whereas plants transferred to soil were kept in the same conditions as at the UiO.

#### 2.1.3 Harvesting genomic plant DNA

The AquaPure Genomic DNA Isolation Kit (Qiagen) was used according to manufacturer's instructions. Plant tissue was collected and kept in liquid Nitrogen, or stored at -80°C.

#### 2.1.4 Isolation of total plant RNA

To prepare RNA for hybridization probes (microarray) and RT-PCR at UCT (*idl5* mutant), total RNA was isolated from 2-week-old seedlings using a method based on the LiCl precipitation method (Verwoerd et al. 1989). About 1 g of tissue per sample was foil-packed

and frozen in liquid N<sub>2</sub>. The frozen tissue was ground in liquid N<sub>2</sub>, and added to a tube containing 3 ml extraction buffer (100mM Tris-HCl, 200mM NaCl, 5mM DTT, 1% Sarcosyl, 20mM EDTA) per gram tissue. 2.8 ml Phenol (pH = 4): chloroform:isoamyl alcohol 50:50:1 was added, and the mixture was centrifuged for 10 minutes (5K, 4°C). The water phase was extracted twice with 3 ml chloroform: isoamyl alcohol 50:1, followed by a centrifugation step for ten minutes (5K, 4°C). Next, 1/3 volume 8M LiCl was added, and the mixture was kept overnight at 4°C in order to precipitate RNA. After spinning for 10 minutes (10K, 4°C), the pellet was dissolved in 1.5mL nuclease-free water and 150µL NaAcetate (3M pH 5.2) and 3.75 mL abs. ethanol were added. The RNA was left to precipitate at -70°C for approximately 1 hour. After centrifugation, the pellet was rinsed twice in cold 70% ethanol and left to dry (37° for 15 minutes) before being dissolved in nuclease-free water. The quality of the total-RNA was assayed on a denaturing agarose gel (see chapter 2.3.1.2).

For RT-PCR performed at the UiO (*IDL4::RNAi*, *35S::IDL4* and *35S::IDL5* constructs), the RNeasy kit (Qiagen) was used according to the manufacturer's instructions.

### **2.1.5 Histochemical GUS assay**

Histochemical assay of GUS-activity (Jefferson 1989) was performed on a selection of plant tissues; whole seedlings, rosette leaves, stem leaves, flowers and mature siliques. The protocol used is adapted from (Schoof et al. 2000) and (Grini et al. 2002). The tissues were incubated in 90% acetone for 10 minutes before being transferred to staining solution (0,05 M NaPO<sub>4</sub>, 0,1% Triton X-100, 2mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O 2mM K<sub>3</sub>Fe(CN)<sub>6</sub>) for an additional 10 minutes. For blue staining by the GUS enzyme, tissues were kept in a substrate solution (0,05 M NaPO<sub>4</sub>, 0,1% Triton X-100, 2mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O 2mM K<sub>3</sub>Fe(CN)<sub>6</sub> and 2mM X-GlcA (Apollo Sci Ltd /Rose Sci Inc.) dissolved in DMF) at 37°C overnight. Following a graded dehydration series to 50% EtOH, the material was post-fixed in FAA (50% EtOH, 10% acetic acid, 5% formaldehyde) for 30 minutes and hydrated in a series of EtOH to 50mM NaPO<sub>4</sub>. The material was mounted on slides in a clearing solution (8:2:1 chloral hydrate:dH<sub>2</sub>O:glycerol) and allowed to clear for 1 hour at 4°C before inspection. This assay is based on the presumption that the fused upstream region of the gene of interest will promote expression of the GUS enzyme. This enzyme will, in turn, hydrolyse X-GlcA to a water-soluble indoxyl intermediate that is further dimerised into a dichloro-dibromo-indigo blue precipitate by an oxidative reaction.

#### **2.1.5.1 GUS-staining of injured leaves**

Premarked rosette leaves from *IDL4::GUS* were cut across the surface with a surgical scalpel. After several time points, leaves were excised and GUS-stained as described above.

### 2.1.6 Transformation of plants with *Agrobacterium tumefaciens* – the floral dip method

This method is based on the *Agrobacterium tumefaciens* ability to integrate its T-DNA from its pTi plasmid randomly into the *Arabidopsis* genome (Bechtold N 1993) modified by Bent and Clough (Clough and Bent 1998). Wild type (ecotype Col-0) plants to be transformed were grown to the flowering stage. In order to obtain more floral buds per plant, the primary bolts were cut 1-3 times. This promotes the proliferation of several secondary bolts.

#### 2.1.6.2 Culturing of *Agrobacterium tumefaciens* and transformation of plants

Cultures of *Agrobacterium* carrying the construct of interest were grown in liquid medium, YEB, containing appropriate antibiotics. The cultures were centrifuged (10 min at 5000 rpm and room temperature) and resuspended in a 5% sucrose solution (made fresh). A surfactant, L-77 (Silwet) was added to a concentration of 0.005%. Whole plants were dipped in the sucrose suspension of *Agrobacterium* cells ( $OD_{600} = 0.8$ ), and left to linger for 2-3 minutes. Dipped plants were placed under a cover to ensure high humidity over night before watered and grown normally. A total of 27 plants were dipped pr construct. Seeds were harvested and grown on plates containing kanamycin to select for transformants.

### 2.1.7 Pathogen infection

Virulent *Pseudomonas syringae* pv. *tomato* was grown overnight at 30°C in KB medium (Peptone 10g/l, Tryptone 10g/l,  $K_2HPO_4$  1.5g/l,  $MgSO_4$  1.5g/l, Glycerol 1%) with 50 µg/ml Rifampicin. The bacteria culture was washed twice in 10mM  $MgCl_2$  and diluted to  $OD_{600} = 0.01$  in 10mM  $MgCl_2$ . The bacterial solution was infiltrated into premarked rosette leaves of 4-week-old *IDL4::GUS* and *IDL5::GUS* plants using a syringe as described in (Glazebrook and Ausubel 1994). Control plants were infiltrated with 10mM  $MgCl_2$ . Cultures of *Botrytis cinerea*, strain brassica, were maintained on apricot halves. Spores were harvested in water and adjusted to a final concentration of  $1-5 \times 10^3$  spores  $ml^{-1}$  using grape juice. Excised leaves from *IDL4::GUS* and *IDL5::GUS* plants were arranged on plates with 0.8% agar, and 3 µl of the spore suspension was placed in the middle of the leaf blade. After being left to dry for 1 hour, the plates' lid were closed to maintain humidity. The plates were kept at 25°C, in 16/8

hours light/ dark cycles. Leaves were monitored daily for development of lesions. Control plants were inoculated with the same volume of grape juice.

### **2.1.8 Segregation analysis**

F2 seeds were harvested from F1 plants (originating from successfully transformed seeds) and grown on MS2 plates containing kanamycin. Seeds that did not develop pass the dicotylous stage were considered to be kanamycin sensitive, thus not containing any T-DNA. Lines with one insertion (multiple insertions of T-DNA are common) were expected to show a 3:1 ratio of kanamycin resistant:sensitive seedlings. A Chi-square test with significance level of 0.05 (i.e.  $\chi^2 = 3.84$ ) was performed to suggest candidate lines.

## **2.2 Bacterial methods**

### **2.2.1 Growth and storage of bacteria**

#### 2.2.1.1 *E. coli*

The *E. coli* strain DH5 $\alpha$  (Invitrogen) was used for amplification and selection of all generated plasmids. In order to produce single colonies, cells were grown on petri dishes containing LA medium (10g/L Bacto tryptone, 5g/L Bacto yeast extract, 0.17M NaCl, 15g/L agar). Cultures were grown in LB medium (LA medium without agar) at 37°C with vigorous shaking (180-200 rpm).

#### 2.2.1.2 *Agrobacterium tumefaciens*

The *Agrobacterium tumefaciens* strain C-58 was utilised for transformation of *Arabidopsis thaliana*. In order to produce single colonies, cells were grown on petri dishes containing solid YEB medium (5g/L Bacto beef extract, 1g/L Bacto yeast extract, 1g/L Bacto peptone, 5g/L sucrose, 15g/L agar, 2ml/L 1M MgSO<sub>4</sub>, pH 7.4). *Agrobacterium* cells were selected with the antibiotics Rifampicin 100 $\mu$ g/mL, Carbenicillin 100 $\mu$ g/mL and a third selection marker in accordance with the introduced plasmid. Liquid cultures were grown in YEB medium without agar at 28°C and vigorous shaking (140rpm).

For prolonged storage, all bacterial cultures were kept in an 8% glycerol solution at -80°C.

## 2.2.2 Transforming bacteria

### 2.2.2.1 Heat-shock method for transforming *E.coli*

*E. coli* strain DH5 $\alpha$  Library Efficient (Invitrogen cat. no 18263-012) and DH5 $\alpha$  Max. Efficient (Invitrogen) were used during the work for this thesis. Transformation was done in accordance with the protocol supplied by the manufacturer.

### 2.2.2.2 Electroporation of *Agrobacterium tumefaciens*

Electroporation is a method used to incorporate exogenous genetic material into bacteria, plant or animal cells. Exogenous DNA is taken up through transient holes in the plasma membrane generated by short, high-voltage, electric impulses. The production of electrocompetent cells and the electroporation was performed in accordance with the protocol equipped with the cuvettes supplied by BioRad (cat. no. 165-2086). Cuvettes with a gap of 0.2 mm were used. After the electroporation, SOC medium (2% bacto trypton, 0.5% Bacto yeast extract, 100mM NaCl, 10mM KCl, 10mM MgCl<sub>2</sub>, 10mM MgSO<sub>4</sub>, 20mM glucose) was added to the cells, and they were allowed to grow at 28°C with shaking for approximately 1 hour. This should be sufficient for the cells to express the resistance genes before they were plated on antibiotic-containing YEB plates.

### 2.2.2.3 Freeze-thaw method for transforming *Agrobacterium tumefaciens*

*Agrobacterium* strain C-58 was grown in YEB medium at 28°C and vigorous shaking until the culture had an OD<sub>600</sub> = 0.5-1.0. 20mM ice cold CaCl<sub>2</sub>, 1 mL pr 50 mL liquid culture, was added to the chilled pellet of the cultures. Aliquots of 0.1mL were made, frozen in liquid nitrogen and stored at -80°C. 100-200 ng exogenous DNA plasmid was added, before thawing the cultures in 37°C water bath for 5 minutes. YEB medium was added to the transformed cells, and they were allowed to grow for 2-4 hours at 28°C with gentle shaking before they were plated on antibiotic-containing YEB plates.

## 2.3 Nucleic acid techniques

### 2.3.1 The Polymerase Chain Reaction (PCR)

PCR (Sambrook and Russel 2001) was used for amplification of specific DNA fragments when cloning, screening for positive bacterial colonies and genotyping plant insertion mutants. Standard set-up was 20 $\mu$ l or 50 $\mu$ l reactions with 200 $\mu$ M dNTP, 0.2 $\mu$ M primers, 1U DNA-polymerase and a suitable amount of template. Dynazyme (Finnenzymes) is a standard

thermostable DNA polymerase suitable for screening and genotyping. Advantage (Clontech) contains a proof-reading enzyme; hence it is more accurate and better suited for cloning. At UCT, the DNA polymerase MY-taq (not commercially available) was used for screening.

### **2.3.2 Agarose gel electrophoresis**

Size determination and separation of DNA fragments were performed by agarose gel electrophoresis (Sambrook and Russel 2001). 1% agarose gels (SeaKem®Leagarose from BioWhittaker Molecule Applications) with ethidium bromide to a final concentration of 0.6µg/ml were run at approximately 80V. The O'GeneRuler 1kb ladder, O'GeneRuler DNA Ladder Mix (both Fermentas), λPstI or λClaI were used to determine the size of DNA fragments.

### **2.3.3 RNA Denaturing gel**

The quality of isolated total-RNA for use in microarray was examined on a denaturing gel (1.2% agarose, 1xMOPS, 6.2% formaldehyde). 2µg RNA was denatured at 65°C for 2.5 minutes and 2x volume loading buffer (1xMOPS, 9%formaldehyde, 60%formamide, 66ng/µl EthBr) was added before the gel was run at 90 V for approximately 1 hour. When assaying total RNA for use in RT-PCR at UiO, a standard 1% agarose gel with EthBr was used (see paragraph 2.3.1).

### **2.3.4 Purification of DNA fragments**

The QIAquick PCR Purification kit available from Qiagen was used according to the manufacturer's specifications.

### **2.3.5 Quantification of DNA and RNA**

DNA was quantified at UiO using the Hoefer DyNAQuant 200 Fluorometer (Amersham Biosciences) as described by the manufacturer. RNA concentration was measured on a Lambda 25 UV/VIS spectrometer (Perkin-Elmer, Inc.). At UCT a ND-1000 Spectrophotometer (NanoDrop Technologies) was utilised to quantify both DNA and RNA.

### **2.3.6 Sequencing DNA**

The automated sequencing reactions are based on the thermal cycle method (Sears 1992). The sequencing is carried out in a similar way to PCR, however only one primer is used, and the



dNTP mix contains a portion of dideoxynucleotides (ddNTP). The four different ddNTPs each carry a specific fluorescent tag. When a ddNTP is incorporated in the growing strand, the synthesis of that particular molecule will terminate. Termination points occur randomly along the length of the template DNA, but are nucleotide specific because of the fluorescent tags. The DNA fragments are separated by capillary electrophoresis, and fluorescent tags are visualised with confocal optics. Sequencing was performed with a MegaBase1000 (Amersham Biosciences) at UiO, or at MWG Biotech, Ebersberg, Germany.

### **2.3.7 Isolation of Bacterial Plasmids, minipreps (Promega)**

Plasmids from *E.coli* and *Agrobacterium tumefaciens* strains were purified from 1.5-4 mL overnight culture as described in the protocol of the Wizard® Plus SV Minipreps DNA Purification System (Promega).

### **2.3.8 Cloning with the TOPO-TA reaction**

TOPO-cloning (Invitrogen) is a system for cloning PCR products. Most of the enzyme mixes designed to generate long PCR products, e.g. Dynazyme, consists mainly of Taq polymerase. This polymerase adds a single deoxyadenosine to the 3' end of PCR products. The TOPO vector is linearised with T overhangs, which facilitates ligation of the PCR products into the vector. The enzyme Topoisomerase I from *Vaccinia* virus is covalently attached to the activated vector, and catalyses incorporation of the PCR product and delineralisation of the vector. The TOPO XL PCR Cloning Kit (Invitrogen) was used according to the manufacturer's protocol. One Shot®TOP10 (Invitrogen) chemically competent *E. coli*, was transformed as described by the manufacturer.

## **2.4 Creating constructs**

### **2.4.1 The Gateway technology**

The Gateway® Technology (Invitrogen) is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda (Landy 1989). A PCR product can be transferred into a donor vector using the BP reaction. The LR reaction facilitates generating an expression clone with the DNA fragment of interest.

### **2.4.2 Creating an entry vector with the BP reaction**

To create expression vectors, such as the RNAi- or over-expression constructs, the gene fragment of interest was amplified by using PCR primers with the attB sequence in their 5'

end. The attB PCR product is transferred to a donor vector, pDONRZeo through the BP reaction. The BP reactions were performed as described in the manufacturer's protocol, however all ingredients were halved and the reactions were incubated at room temperature overnight. DH5 $\alpha$  Library Efficient (Invitrogen) or DH5 $\alpha$  Max Efficient (Invitrogen) chemically competent cells were transformed with the reactions.

#### **2.4.3 Transferring a sequence to a destination vector with the LR reaction**

An entry clone contains a cloned fragment flanked by attL sites. These sites facilitate the introduction of the fragments into destination vectors containing the attR sites. The destination vectors for the RNAi-and over expression constructs were pHELLSGATE8 (CSIRO) and pK7WG2 (Karimi et al. 2005), respectively. As for the BP reaction, the ingredients were halved compared to the protocol and the reactions were incubated at room temperature overnight. The completed reactions were used to transform DH5 $\alpha$  Library Efficient (Invitrogen) or DH5 $\alpha$  Max Efficient (Invitrogen) chemically competent cells.

#### **2.4.4 The RNAi gene silencing vectors**

Standard PCR reactions with the primers IDL4attB1/ IDL4attB2 and IDL5attB1/IDL5attB2 were used to obtain fragments of DNA corresponding to the respective *IDL* genes with terminal attB sites. The attB primers contain a point mutation that results in disruption of the gene's stop codon in the PCR products. The PCR products were purified and recombined into the pDONRZeo (Invitrogen) using the BP reaction. The recombination procedure was performed according to the manufacturer's instructions, using half the volume recommended in the protocol. The BP reaction mix was transformed into chemically competent DH5 $\alpha$  Library efficient cells (Invitrogen). Colonies were collected, and plasmid minipreps of the entry clones from 5 ON cultures were isolated. To investigate whether the recombination had been successful, overnight cultures were screened by PCR with the primers HU and HR together with the gene specific IDL4R and IDL5L. An LR reaction was performed to recombine the *IDL* fragment from its entry clone into the pHELLSGATE8 destination vector (CSIRO). The BP reaction mix was transformed into chemically competent DH5 $\alpha$  Library efficient cells (Invitrogen). Colonies were collected, and plasmid minipreps of the entry clones from 5 ON cultures were isolated. The minipreps were screened for positive recombinants by PCR with the same primer combination as described for the DH5 $\alpha$ . Positive entry clones consist of Ti plasmids with two copies of the gene of interest, separated by an intron, in front of the

constitutive 35S promoter. When expressed in transformed plants, the construct will encode hairpin RNA as a possible trigger for targeted gene silencing.

#### 2.4.5 The 35S over expression vectors

Standard PCR reactions with the primers IDL4attB1stop/IDL4attB2stop and IDL5attB1stop/IDL5attB2stop were used to obtain fragments of DNA corresponding to the respective *IDL* genes with terminal attB sites. Recombination with the BP reaction was performed as previously described, and the BP reaction mix was transformed into chemically competent DH5 $\alpha$  Max efficient cells (Invitrogen). The LR reaction was performed with the entry clones and the destination vector pK7WG2 (Karimi et al. 2005) as described in chapter 2.4.4, followed by transformation into chemically competent DH5 $\alpha$  Max efficient cells (Invitrogen). The expression clones were confirmed with DNA sequencing using the primer 35SL. The resulting vectors are Ti plasmids encoding the genes *IDL4* and *IDL5* controlled by the constitutive 35S promoter from *Cauli flower Mozaic Virus*. This should lead to abnormally high expression levels of the genes in all tissues of the transformed plants.

### 2.5 Microarray analysis

Microarray analysis was performed to screen for molecular phenotypes in the *idl5* mutant, i.e. screen for differentially expressed genes in seedlings between wildtype and mutant. cDNA from mutant is labelled with one fluorescent colour, and cDNA from wildtype with another. Both samples are hybridised to an oligonucleotide microarray slide, and fluorescence levels are read and compared for each oligonucleotide element. All *Arabidopsis* genes that have standard ORF names (e.g. At2g01130) are represented with one or more spots on the slides.

#### 2.5.1 the Microarray slides

The microarray experiments were performed at the CAPAR facility at the University of Cape Town, South Africa. The slides were glass microarrays containing 29,000 70-mer-oligonucleotide elements for *Arabidopsis thaliana* provided by The University of Arizona. (Galbraith 2005). In order to immobilise the oligonucleotide array elements, the slides were rehydrated, UV-cross linked and washed as described in the provider's manual.

#### 2.5.2 Synthesis of aminoallyl-labelled cDNA

As described in CAPAR's protocol (van Dyk 2005), SuperScript III Reverse Transcriptase (Invitrogen) and AA-dUTP (Sigma) were used to synthesise aminoallyl-labelled cDNA from

total plant RNA. AA-cDNA was coupled to either Cyanine 3 or 5 (Cy3/Cy5, AmershamPharmacia), and purified with the RNeasy Mini kit (Qiagen).

#### 2.5.3 Pre-hybridisation of and hybridisation onto the microarray slides

The slides were treated with a prehybridisation buffer containing SSC, SDS and BSA as explained in the CAPAR protocol (van Dyk 2005). This step is performed to minimise unspecific binding to the probes. Equal amounts of Cy3 and Cy5-labelled cDNA were applied to the slides as described in the CAPAR protocol. The hybridisation was performed in a water bath at 42°C for approximately 18 hours.

#### 2.5.4 Washing and scanning the hybridised microarrays

The hybridised slides were washed in a series of decreasing SDS and SSC concentrations. Refer to the CAPAR protocol. Following centrifugation at 1000rpm for 5 minutes, the slides were scanned with an Axon 4000B dual-laser scanner. The scanner was operated through the computer software GenePix 5.0 Pro (Axon Instruments). The scanner has two lasers that simultaneously scan at 532nm and 635nm (635nm for Cy5 red and 532nm for Cy3 green). The PMT (photo multiplier tube) settings for the two fluorophores were adjusted, so that the two frequency histograms could be set to overlap as much as possible.

### **2.6 Bioinformatic analysis**

Primers were designed with the software VectorNTI v. 8-9 (InforMax). For nucleotide sequence alignment, the application ContigExpress was used. VectorNTI v. 10 (InforMax) was used to perform the peptide sequence alignment in section 1.8.2. The software uses the ClustalW algorithm for alignments. In order to find putative insertion mutation lines of a given sequence, the universal BASIC Local Alignment Search Tool (BLAST) engine (Altschul et al. 1990) was utilised at the SALK insertional mutagenesis database <http://signal.salk.edu/cgi-bin/tdnaexpress>.

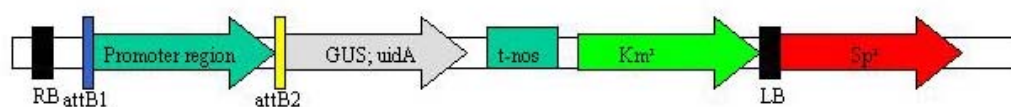
### 3 RESULTS

In this project, the *Arabidopsis* genes *IDL4* and *IDL5* have been examined for expression pattern and function. Transgenic plants harbouring a promoter::GUS construct were used to investigate promoter activity. A putative loss-of-function mutant for *IDL5* was identified and further investigated. Since no mutant line with insertion situated in the promoter region or cds of *IDL4* was found, we generated RNAi gene silencing lines for this gene. Plants over expressing the *IDL* genes were constructed, and screened for any gain-of-function phenotypes.

#### 3.1 Analysis of promoter activity – the GUS assay

Prior RT-PCR experiments (Butenko et al. 2003) showed that *IDL4* and *IDL5* are expressed in a diversity of tissues. The authors noted that both of these genes seemed to be expressed at higher relative levels in floral organs. *IDL4* mRNA was also shown in roots, and *IDL5* mRNA was shown in seedlings.

The GUS-assay is a method for studying the expected localization of expression in more detail. The promoter region of a gene of interest is cloned downstream of a promoter less reporter gene,  $\beta$ -glucoronidase (*GUS*). The *GUS* expression is thus regulated as the gene under investigation. The *IDL4*::*GUS* and *IDL5*::*GUS* constructs were made and transformed into *Arabidopsis* Col-0 plants by Melinka Butenko (2003, unpublished results). A 1980 and 2020 base pair region upstream of *IDL4* and *IDL5*, respectively, were cloned upstream of the promoter less *GUS* gene in the binary vector pPZP211G-GAWI (figure 3.1).



**Figure 3.1 The promoter::GUS reporter gene constructs**

The expected promoter region of *AtIDL4* and *AtIDL5* were cloned in front of the *GUS* reporter gene in the vector pPZP211G-GAWI. The putative promoter region included 1980 and 2020 base pairs upstream of *IDL4* and *IDL5* respectively. The vector additionally harbours a *nos* terminator, *nptII* (*Km<sup>r</sup>*) and the right- and left border sequences.

##### 3.1.1 Segregation analysis: Identification of single insertion lines

One would expect lines with only one copy of the promoter::*GUS* T-DNA to be less prone to transgenic silencing, compared to lines with several copies of the insert. Therefore, a

segregation analysis of T<sub>2</sub> seeds was performed. The offspring of a self-fertilising hemizygous single copy *nptII* plant will show a 3:1 mendelian distribution between Km<sup>r</sup> and Km<sup>s</sup> plants. Seeds harvested from individual T<sub>1</sub> GUS plants were grown on medium containing kanamycin. After two weeks, seedlings were scored as being Km<sup>r</sup> or Km<sup>s</sup>. The segregation numbers were evaluated with a chi-square significance test:

$$\chi^2 = \sum \frac{(F_o - F_e)^2}{F_e}, \text{ where } F_o \text{ is observed frequency, and } F_e \text{ is expected frequency}$$

For lines with a  $\chi^2$  less than 3.84 the hypothesis holds with at least 95% accuracy and thus the hypothesis of a 3:1 distribution would not be rejected in these cases. Results are shown in table 3.1 and 3.2.

**Table 3.1 Segregational analysis of *IDL4::GUS* T<sub>2</sub> plants**

GUS Line	Observed Km <sup>r</sup>	Observed Km <sup>s</sup>	Expected Km <sup>r</sup> 3:1	Expected Km <sup>s</sup> 3:1	$\chi^2$
IDL4-1	56	2	43.50	14.50	14.400
<b>IDL4-2</b>	<b>61</b>	<b>24</b>	<b>63.75</b>	<b>21.25</b>	<b>0.475</b>
IDL4-4	82	0	61.50	20.50	27.333
<b>IDL4-5</b>	<b>59</b>	<b>25</b>	<b>63.00</b>	<b>21.00</b>	<b>1.000</b>
<b>IDL4-6</b>	<b>77</b>	<b>23</b>	<b>75.00</b>	<b>25.00</b>	<b>0.213</b>
IDL4-8	60	0	45.00	15.00	20.000
<b>IDL4-10</b>	<b>83</b>	<b>29</b>	<b>84.00</b>	<b>28.00</b>	<b>0.000</b>
<b>IDL4-12</b>	<b>72</b>	<b>32</b>	<b>78.00</b>	<b>26.00</b>	<b>1.800</b>
<b>IDL4-14</b>	<b>54</b>	<b>14</b>	<b>51.00</b>	<b>17.00</b>	<b>0.700</b>
IDL4-15	22	0	16.50	5.50	7.333
<b>IDL4-16</b>	<b>68</b>	<b>17</b>	<b>63.75</b>	<b>21.25</b>	<b>1.100</b>
<b>IDL4-17</b>	<b>95</b>	<b>40</b>	<b>101.25</b>	<b>33.75</b>	<b>1.543</b>
<b>IDL4-18</b>	<b>94</b>	<b>40</b>	<b>100.50</b>	<b>33.50</b>	<b>1.700</b>
<b>IDL4-20</b>	<b>83</b>	<b>35</b>	<b>88.50</b>	<b>29.50</b>	<b>1.367</b>
IDL4-21	70	0	52.50	17.50	23.300
IDL4-22	71	9	60.00	20.00	8.067
<b>IDL4-23</b>	<b>55</b>	<b>14</b>	<b>51.75</b>	<b>17.25</b>	<b>0.800</b>
IDL4-24	92	8	75.00	25.00	15.413
IDL4-26	50	29	59.25	19.75	5.776
IDL4-27	94	6	75.00	25.00	19.300
IDL4-28	52	37	66.75	22.25	13.037
IDL4-29	55	5	45.00	15.00	8.900
<b>IDL4-31</b>	<b>56</b>	<b>26</b>	<b>61.50</b>	<b>20.50</b>	<b>1.967</b>
IDL4-32	60	0	45.00	15.00	20.000
<b>IDL4-33</b>	<b>63</b>	<b>19</b>	<b>61.50</b>	<b>20.50</b>	<b>0.146</b>
IDL4-34	60	5	48.75	16.25	10.400
IDL4-35	70	2	54.00	18.00	18.963

**Table 3.2 Segregational analysis of *IDL5::GUS* T<sub>2</sub> plants**

GUS Line	Observed Km <sup>r</sup>	Observed Km <sup>s</sup>	Expected Km <sup>r</sup> 3:1	Expected Km <sup>s</sup> 3:1	$\chi^2$
IDL5-2	1	120	90.8	30.3	355.044
IDL5-3	105	10	86.3	28.8	16.304
<b>IDL5-4</b>	<b>104</b>	<b>28</b>	<b>99.0</b>	<b>33.0</b>	<b>1.010</b>
<b>IDL5-5</b>	<b>102</b>	<b>36</b>	<b>103.5</b>	<b>34.5</b>	<b>0.087</b>
IDL5-6	150	4	115.5	38.5	41.221
<b>IDL5-8</b>	<b>160</b>	<b>51</b>	<b>158.3</b>	<b>52.8</b>	<b>0.077</b>
<b>IDL5-9</b>	<b>42</b>	<b>12</b>	<b>40.5</b>	<b>13.5</b>	<b>0.222</b>
<b>IDL5-10</b>	<b>140</b>	<b>61</b>	<b>150.8</b>	<b>50.3</b>	<b>3.066</b>
IDL5-11	150	18	126.0	42.0	18.286
IDL5-12	200	14	160.5	53.5	38.885
IDL5-13	200	20	165.0	55.0	29.697
<b>IDL5-15</b>	<b>180</b>	<b>60</b>	<b>180.0</b>	<b>60.0</b>	<b>0.000</b>
<b>IDL5-16</b>	<b>220</b>	<b>58</b>	<b>208.5</b>	<b>69.5</b>	<b>2.537</b>
IDL5-17	180	3	137.3	45.8	53.262
<b>IDL5-18</b>	<b>180</b>	<b>47</b>	<b>170.3</b>	<b>56.8</b>	<b>2.233</b>
<b>IDL5-19</b>	<b>150</b>	<b>45</b>	<b>146.3</b>	<b>48.8</b>	<b>0.385</b>
IDL5-20	50	80	97.5	32.5	92.564
IDL5-21	120	7	95.3	31.8	25.724
<b>IDL5-22</b>	<b>180</b>	<b>61</b>	<b>180.8</b>	<b>60.3</b>	<b>0.012</b>

**Table 3.1 and 3.2 Segregation numbers of promoter-GUS transformants.**

The expected number of kanamycin resistant and kanamycin sensitive plants were computed according to the 3:1 segregation hypothesis. The hypothesis was tested with the chi-square method,  $\chi^2$  critical value 3.84. The tables show the lines where the hypothesis holds with at least 95% accuracy in bold. Km<sup>r</sup>: kanamycin resistant plants. Km<sup>s</sup>: kanamycin sensitive plants.

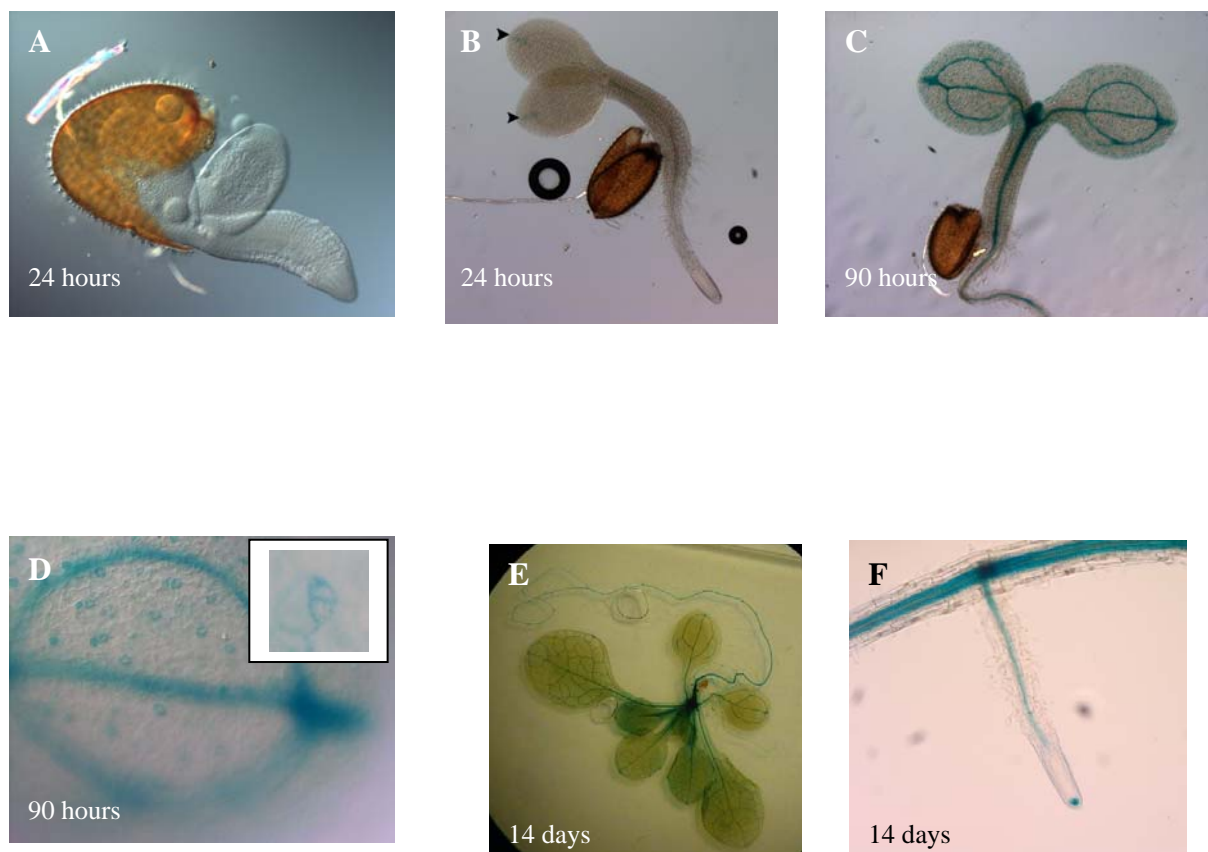
### 3.1.2 *IDL4*-promoter *GUS* expression pattern

Tissues from the expected one-copy lines (generation T<sub>2</sub>) *IDL4::GUS* -2, 5, 10, 12, 17, and 20 were fixated and stained with X-gluc solution overnight. *GUS* expression was detected in all of the tested lines. The lines showed a varying degree of staining, but for most of the lines, blue colour was seen both in seedlings and adult tissues. The expression pattern in seedlings included most distal vascular tissue of cotyledons shortly after emergence from the seed coat, guard cells of younger seedlings and vascular tissue including hydathodes (fig 3.2). *GUS* activity was evident in the vascular tissue of both primary and lateral roots, but cells in the root tip stained solely in lateral roots (fig 3.3). *GUS* expression in the inflorescence (fig 3.4) was apparent in the branching point between stamen and leaves, floral abscission zone and vascular bundles at the base of siliques, the style of siliques and the carpel valves. *GUS* expression was also seen in the funicle of mature siliques, which is the stalk of the seeds. Staining was observed in some hydathodes of adult rosette and cauline leaves, but was not

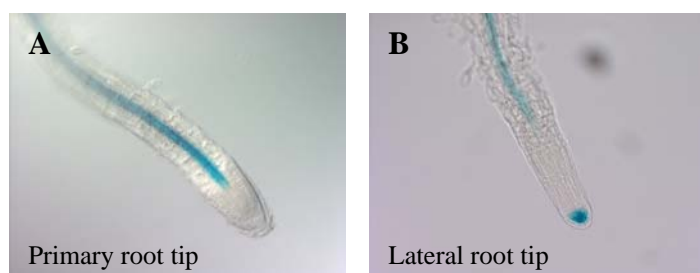
apparent in roots of adult plants. Line *IDL4::GUS* 12, which is depicted in figure 3.2-4, showed an intermediate staining intensity.

**Fig 3.2 *IDL4* promoter –driven GUS expression in seedlings**

*IDL4* promoter::*GUS* shows GUS expression in the most distal vascular parts of cotyledons shortly after emergence from seed coat (B), in vascular tissue and guard cells of young seedlings (C and D) and in vascular tissue of developed seedlings (E and F). GUS expression could also be seen in cells immediately adjacent to developing stomata (C, insert). Timepoints refer to period in light.





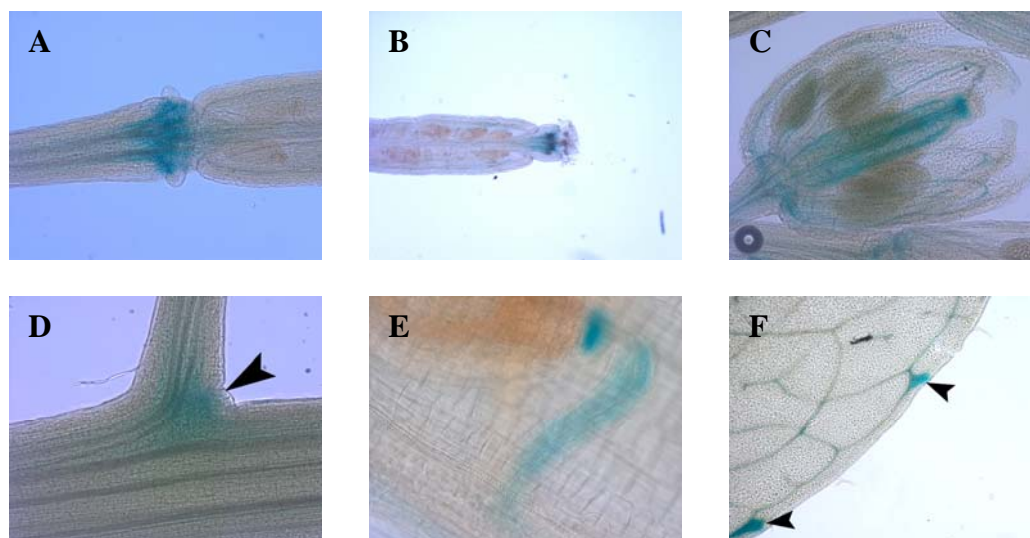


**Fig 3.3 Comparison of primary and lateral root tips**

The GUS expression in primary roots was restricted to the differentiation zone (A), but appeared in the meristematic parts as well in lateral roots (B)

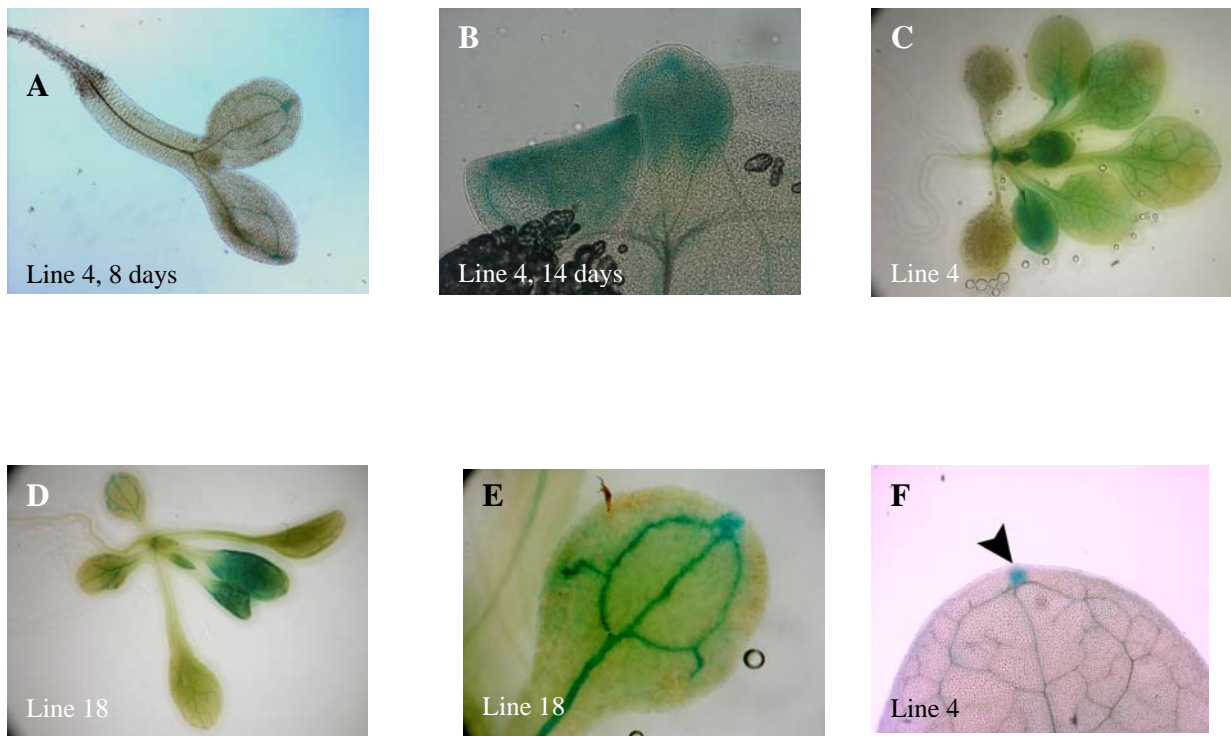
**Fig 3.4 *IDL4* promoter –driven GUS expression in adult plants**

The *IDL4* promoter directs GUS expression in the floral abscission zone/ vascular bundles at the base of siliques (A), in the style (B), valves of developing carpels (C), at the adaxial leaf axil (D), in the funicles and seed abscission zone (E). (F) shows GUS expression in hydathodes of an adult cauline leaf.



### 3.1.3 *IDL5* *GUS* expression

Tissues from the expected one-copy lines (generation T<sub>2</sub>, segregating 3:1) *IDL5::GUS* 4, 8, 10, 15, 16, 18 and 19 were fixated and stained with X-gluc solution overnight. *GUS* expression was detected in three of the lines, namely *IDL5::GUS* 4, 16 and 18. Line 16 showed a weaker expression than the two others, and *GUS* staining was only visible in 1 of 4 individuals stained. In seedlings, *GUS* expression was visible in leaves and hypocotyl, with a tendency to be concentrated in vascular tissue (Fig 3.5 C) and hydathodes (Fig 3.5 D). No staining was observed in roots. Apart from very weak *GUS* expression in hydathodes of cauline- and rosette leaves, no blue staining was observed in adult tissues (data not shown).



**Figure 3.5 *IDL5* promoter –driven *GUS* expression**

The *IDL5* promoter directs *GUS* expression in above-ground parts of seedlings some time after the cotyledons are stretched out (A). (B) depicts occurrence of the second pair of true leaves. The reporter gene activity continues through the leaf development stage; (C) and (D), and is mostly concentrated in vascular tissue and hydathodes; (E) and (F), respectively.

### 3.1.4 Further analysis of *GUS* expression

One of the patterns that the two promoter::*GUS* constructs have in common is the activity in hydathodes. As discussed in the introduction (chapter 1.5.1) hydathodes are consistently open pores that may function as point of entry for various pathogens. Several genes involved in defence responses are expressed in hydathodes (e.g. (Chen et al. 2003; Kobae et al. 2006)), therefore we investigated whether the promoter of *IDL4* or *IDL5* would be further activated upon infection with pathogens.

#### 3.1.4.1 *GUS* expression induced by pathogens

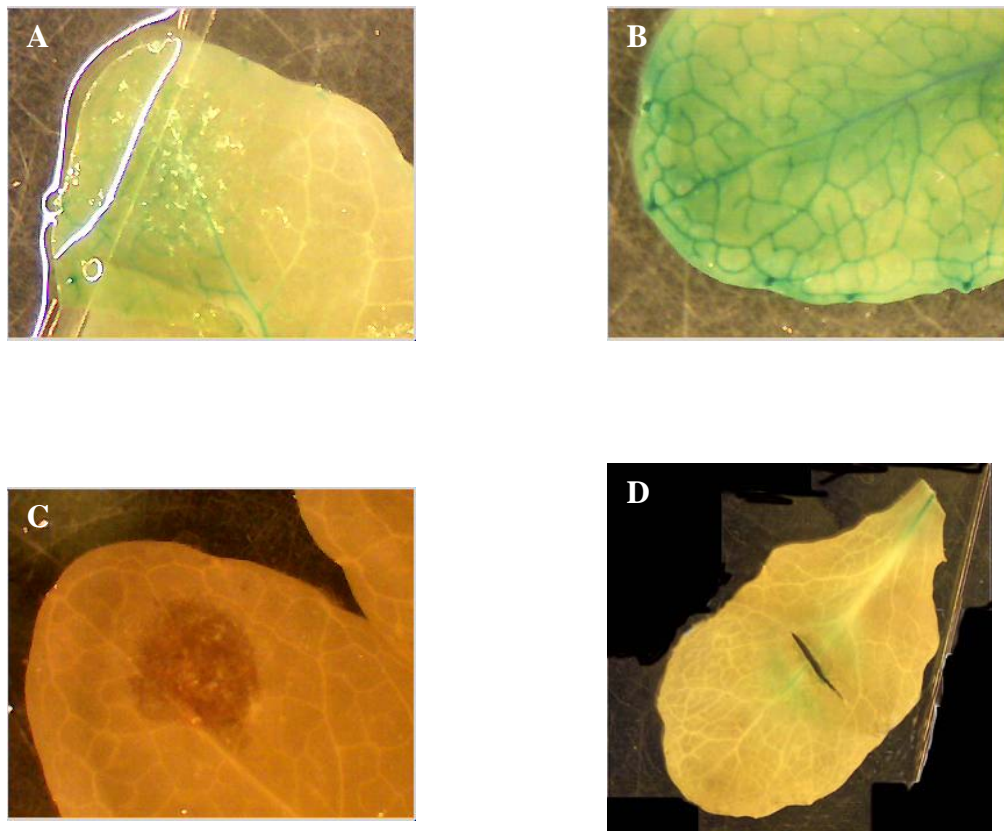
In this analysis a bacteria suspension of virulent *P. syringae* pv. *tomato* DC3000 was syringe-infiltrated into the intercellular space of 4-week old *IDL4*::*GUS* and *IDL5*::*GUS* rosette leaves. At this age there was no intrinsic *GUS* activity seen in any of the two constructs. The infected tissue was fixated and stained in X-gluc solution overnight at time points 6-24 hours post-infection. There was no apparent correlation between *P. syringae* infection and *GUS* expression. Some *IDL4*::*GUS* leaves showed *GUS* expression when injected with either bacterial suspension or the sterile control solution (blue areas depicted in Figure 3.6 B and C), whereas others did not (results not shown). A possible explanation of the observed *GUS* expression could be that the *IDL4* promoter is activated in a wounding response to the injection itself.

A suspension of spores from the necrotrophic pathogen *Botrytis cinerea* was applied to detached *IDL4*::*GUS* and *IDL5*::*GUS* rosette leaves. The leaves were kept on agar until lesions were visible (24 hours), fixated and stained as described above. The transformants did not show *GUS* staining upon infection with the necrotrophic pathogen, *B. cinerea*, shown for *IDL4* in figure 3.6 D.

#### 3.1.4.2 *GUS* expression induced by injury

Based on the inconsistent data from the experiments with *P. syringae* infection, we further investigated whether the promoter of *IDL4* is activated during an injury response in *Arabidopsis*. *IDL4*::*GUS* rosette leaves were injured before staining with X-gluc. Leaves were cut with a surgical blade, up to 12 hours prior to staining. Some of the leaves treated in the initial experiments showed reporter gene activity in vascular tissue surrounding the wounding site (blue areas depicted in Figure 3.7 A). However, when repeated, we did not see the same tendency. Note that there is *GUS* expressed at the base of the leaf as well. It is

highly unlikely that this come from a wounding response, as the leaf is cut off immediately before fixation and staining.



**Figure 3.6 GUS expression in infected and injured *IDL4::GUS* leaves**

(A) *IDL4::GUS* injected with bacterial suspension (B) *IDL4::GUS* injected with sterile  $\text{MgCl}_2$  (aq) control (C) *Botrytis cinerea*-infected *IDL4::GUS* rosette leaf, 24 hours post infection (D) Injured *IDL4::GUS* rosette leaf

### 3.2 Obtaining and investigating lack-of-function mutants

#### 3.2.1 Obtaining insertion mutants

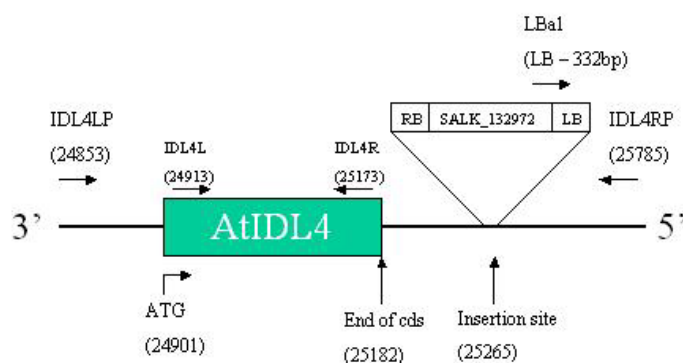
In order to determine the function of a specific gene, it is useful to identify a loss-of-function mutation in the given gene. One can thus compare plants that carry this mutation with wild type plants to detect any mutant phenotypes. One way of disrupting a gene's expression is by having a sequence of foreign DNA inserted within the gene. Several databases, e.g. the Signal SALK database (<http://signal.salk.edu/cgi-bin/tdnaexpress>), with collections of such insertion lines, exist. These databases can be screened *in silico* with BLAST searches against a query sequence. Seeds of putative mutants can be ordered through the *Arabidopsis* stock centers (<http://www.Arabidopsis.org/links/atlinks.jsp>) for further studies.

The Signal SALK database was BLAST searched using the known *IDL4* genomic sequence (Melinka Butenko). A SALK line, SALK\_132972, with a T-DNA insertion close to the coding region of *IDL4* was identified. The Salk Institute (<http://signal.salk.edu/cgi-bin/tdnaexpress>) suggests that the exact position of a given T-DNA insertion is within 300bp from the site proposed in their database. Thus it was necessary to verify whether the insertion was indeed within the coding sequence (cds) of the gene, particularly since the *IDA-like* genes have relatively short sequences. Furthermore, most genes show the wildtype phenotype if the plants are hemizygous for a mutation, and it is therefore desirable to obtain homozygous plants before screening for mutant phenotypes.

The Signal SALK database was also BLAST searched using the known *IDL5* genomic sequence (Melinka Butenko). A SLAT (Sainsbury Laboratory *Arabidopsis thaliana* Transposants) line, SM\_3.5361, was found likely to have an insertion situated within the cds of the *IDL5* gene. As for the putative *IDL4* insertion line, homozygous SM\_3.5361 plants were obtained and further investigated.

### 3.2.2 Investigating the SALK\_132972 insertion line

Ragnhild Nesteskog, NARC, obtained homozygous SALK\_132972 lines, which were further analyzed. The progeny of 6 homozygous T<sub>3</sub> plants were grown on selective medium and screened for aberrant phenotypes. No such phenotypes were discovered under normal growth conditions. Genomic DNA was isolated from two of the homozygous lines, and used to verify the position of the insertion. The sequence between the insertion and flanking region was amplified with PCR using the primers LBa1 (insertion) and IDL4RP (downstream of the *IDL4* gene). The resulting PCR product (~850 bp) was sub-cloned and sequenced. Alignment of the flanking sequence to the *Arabidopsis* genome, revealed that the insertion was situated 83 base pairs down stream of the *IDL4* cds (fig 3.7).



**Fig 3.7 *IDL4* and its putative insertion**

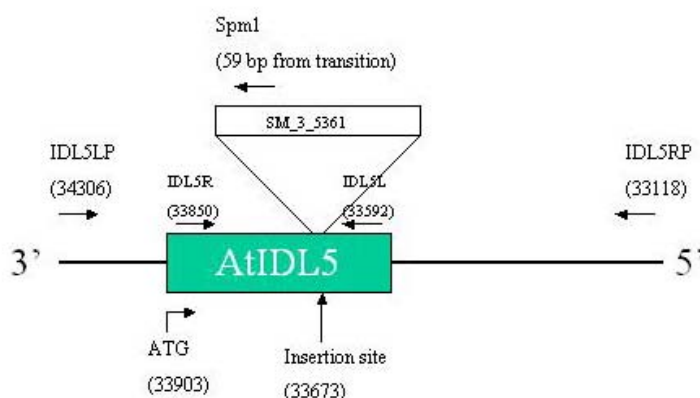
The *IDL4* gene and the SALK\_132972 insertion, with primers used in the experiments. Numbers refer to clone Chr.3 MVE11. The drawing is not to scale.

### 3.2.3 Investigating the SM\_3.5361 insertion line

Seeds from the SM\_3.5361 (hereafter called *idl5spm*) line were ordered from the stock center NASC (<http://Arabidopsis.info/>), and a total of 32 plants were grown without selective medium. Leaves were collected by M.A. Butenko and genomic DNA was isolated. The DNA from the T<sub>2</sub> generation was used to confirm the position of the insertion. Genomic DNA from the T<sub>3</sub> generation was used to identify homozygous plants for further studies.

#### 3.2.3.1 Verifying the position of the dSpm element

The 3' flanking region of the insertion in *idl5spm* was amplified using PCR with the primers Spm1, located within the insertion, and IDL5LP, from a region upstream of the *IDL5* gene, fig 3.8. The PCR amplified a fragment of approximately 700 bp, which was further subcloned and sequenced. Alignment of the sequence to the *Arabidopsis* genome verified the position of the dSpm element to be within the cds of the *IDL5* gene. See fig 3.8.



**Figure 3.8 *IDL5* and its insertion**

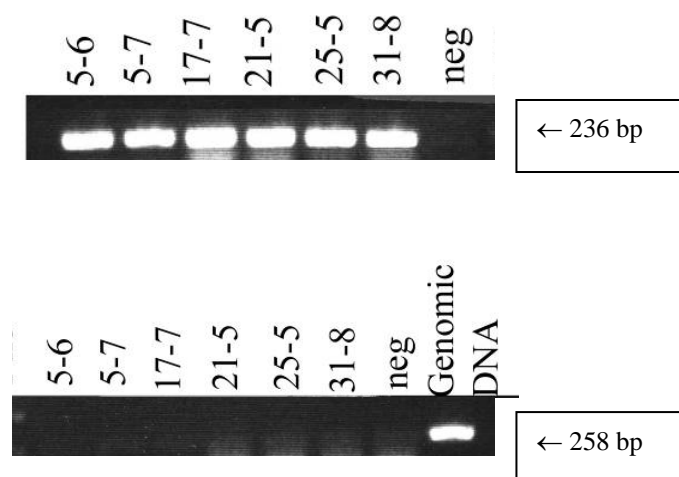
Position of the SM\_3.5361 insertion. The figure shows part of *Arabidopsis* chromosome 1 (numbers refer to clone BAC F22K20), which includes the gene *IDL5*. Location of the insertion SM\_3.5361 and primers used for genotyping and subcloning the border sequences are shown. The drawing is not to scale.

#### 3.2.3.2 Genotyping *idl5* insertion mutants

In order to obtain homozygous lines, PCR with the insertion-specific primer spm1 and the gene-specific primers IDL5L and IDL5R was used to find individual plants homozygous for the insertion in the *IDL5* gene.

The PCR reaction 1 with the two gene-specific primers (fig 3.9 B) would yield a fragment of approximately 258 bp if either of the chromosome pairs lacks the insertion. If there indeed is an insertion, the region between the two primers would increase by ~6kb, making an

amplified fragment unlikely with the extension time used. The primers Spm1 and IDL5R would amplify a fragment of about 236 bp if a dSpm insertion was present in reaction 2 (fig 3.9 A). Thus a wild type plant would render a PCR product exclusively in reaction 1, whereas for homozygous mutant plants only reaction 2 would give a product. Hemizygous plants would yield products in both reaction 1 and 2.



**Fig 3.9 Genotyping progeny of the *idl5* dSpm insertion line**

**A:** PCR products with the primers spm1 (insertion) and IDL5R (gene-specific) on genomic DNA obtained from T3 plants of the insertion line.

**B:** PCR products with the gene-specific primers IDL5L and IDL5R. None of the DNA samples show the wildtype band (refer to wt control). The results suggest that these plants are homozygous for the insertion (see text for details).

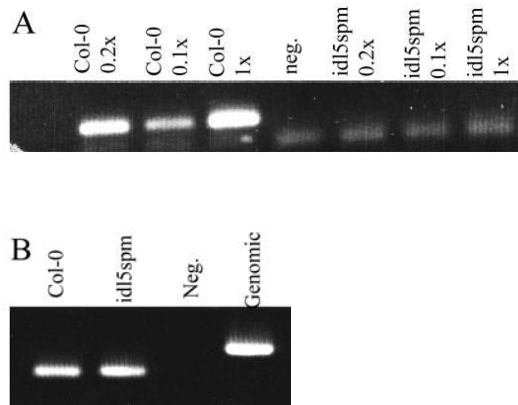
Of the of the 32 T3 plants genotyped, 6 were shown to be homozygous for the dSpm insert. Progeny from the homozygous plants were grown, but no visible phenotype was detected. The lines 5, 21 and 25 were randomly picked for further work.

### 3.2.3.3 Reverse-Transcriptase PCR

Since no mutant phenotype was observed in *idl5spm* homozygous plants, Reverse Transcriptase PCR (RT-PCR) was performed to see if a reduction of the *IDL5* mRNA could be detected. Previous RT-PCR (Butenko et al. 2003), and preliminary GUS-assay results (see chapter 3.1) suggested that seedlings between the age of 7 and 14 days would be likely candidates for detecting *IDL5* transcript in wild type plants. First-strand cDNA was made from total-RNA originating from ~50 whole seedlings at the age of 12 days (line 21-5). The *IDL5* sequence does not contain any introns, hence the cDNA was checked for genomic DNA contamination with intron-spanning ubiquitin primers (figure 3.10 B). PCR with gene-specific



primers AtIDL5R and AtIDL5L was performed on the cDNA. The RT-PCR shows reduction of *IDL5* mRNA in the mutant line tested, 21-5. (fig 3.10 A)



**Fig 3.10 RT-PCR on *idl5* seedlings**

RT-PCR could not detect *IDL5* transcript in the insertion line 21-5.

**A.** PCR with gene-specific primers *IDL5L* and *IDL5R* on various amounts of cDNA from both wild type (*Col-0*) and *idl5* tissue. PCR was run at 36 cycles, however *IDL5* cDNA was detectable at 30 cycles in the wild type sample (data not shown). All bands 258 bp.

**B.** PCR with intron-spanning *UBIQUITIN* primers detects no genomic contamination in either sample.

### 3.3 The RNA interference assay

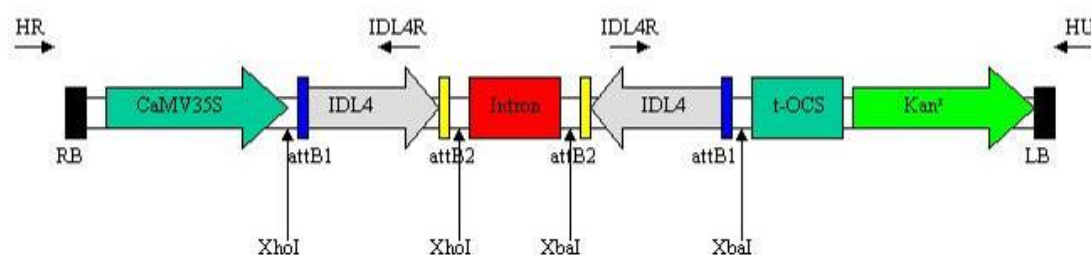
In the SALK insertion line for *IDL4*, the T-DNA was located downstream of the gene, and no abnormal phenotype was observed. Therefore, RNA interference (RNAi) was used to create knockdown lines with decreased *IDL4* expression, and these RNAi lines were screened for mutant phenotypes.

#### 3.3.1 The RNAi constructs

The RNAi constructs were made with the Gateway cloning technology, as described in chapter 2.4.4. The entry clones included the whole genome sequence of the *IDL4* gene, with a point mutation disrupting the stop codon. If a stop codon was present, it would disrupt the continuous expression of the construct *in planta*. The entry clone was recombined with the destination vector pHELLSGATE8 (Csiro) (Wesley *et al.* 2001). pHELLSGATE8 has two destination sites, and the gene is introduced in duplicate as an inverted repeat, separated by an intron (fig 3.11). When the construct is expressed *in planta*, mRNA will form a hairpin structure with the intron spliced out. This double stranded RNA triggers the breakdown of sequence-specific mRNA, thus causing a decrease in the amount of translated protein. *E.coli*

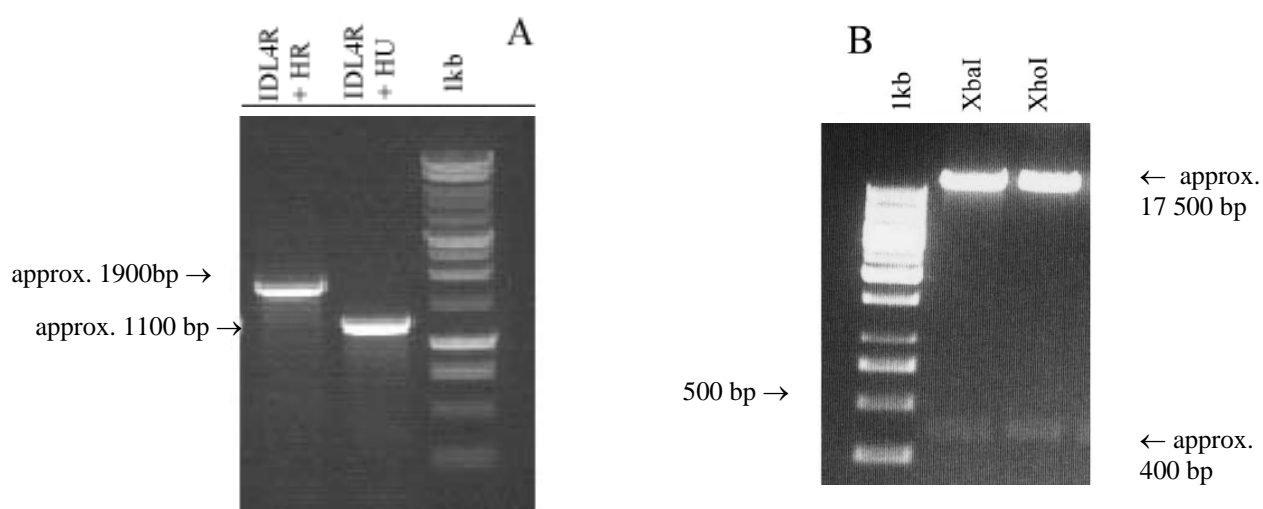


colonies transformed with the expression clones were screened with PCR and digested to identify correct insertions (fig 3.12 A). Plasmid minipreps were digested with restriction endonucleases to confirm that the recombined fragments were in the correct orientation (fig 3.12 B). Following transformation into *Agrobacterium*, PCR testing was repeated. A positive *Agrobacterium* clone was used to transform wildtype *Arabidopsis* Col-0 plants. Six transformants were obtained for the T<sub>1</sub> generation.



**Fig 3.11 IDL4 RNAi construct**

The part of the pHELLSGATE8 vector transferred to plants. In addition to Right and Left Borders to aid transformation, the construct bears Kanamycin resistance (the *nptII* gene with promoter and terminator). The sequence that is to form the hair pin RNA, is driven by the constitutive promoter, CaMV35S. t-OCS is the OCS terminator. Vertical arrows indicate restriction sites for the relevant enzymes.



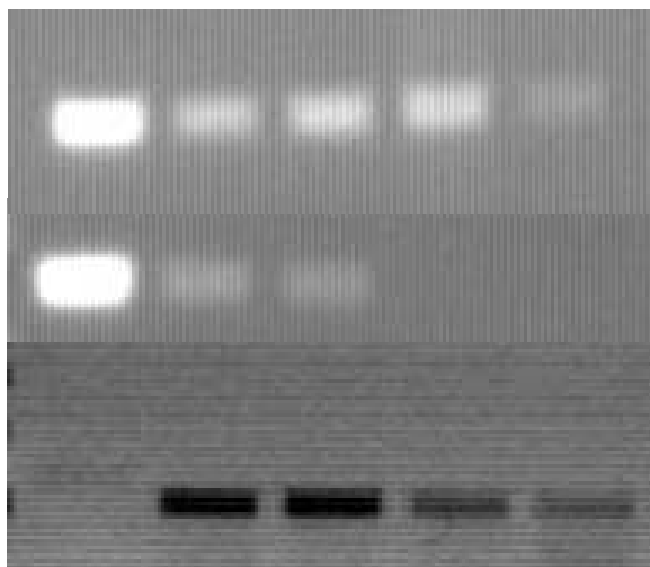
**Fig 3.12 Making of the RNAi constructs**

**A.** PCR-testing of an *Agrobacterium* culture of bacteria harbouring the expression clone pHELLSGATE8. The results show bands from primers IDL4R/ HR and IDL4R/ HU. The ladder is 1kb (Fermentas). IDL4R/ HR amplifies a fragment of ~1900 bp. IDL4R/ HU amplifies a fragment of ~1350 bp. The resulting bands correspond to expected fragment lengths if the *IDL4* sequence is present in the correct orientation (1920 and 1369 bp, respectively). **B.** The plasmid from *E.coli* was cut with the restriction enzymes XbaI and XhoI. The resulting bands are of the expected sizes. If the intron was inverted, the fragments would be larger.

### **3.3.2 Semi-quantitative RT-PCR of *IDL4::RNAi* transformants**

RT-PCR is a method to investigate the relative expression levels of genes. 2-week old seedlings and buds/ young flowers from *IDL4::RNAi* plants were chosen for the study, based on the results from promoter::*GUS* analysis (chapter 3.1) and former RT-PCR (Butenko et al. 2003). Total RNA was isolated from pooled individuals of separate T3 lines. cDNA was synthesised using equal amounts of total-RNA from both wild type and *IDL4::RNAi* tissues. The primers that were used in the experiment were designed to amplify a region including parts of the 5' and 3'untranslated regions (UTR). This was to ensure that any amplification was restricted to the endogenous *IDL4* RNA, and not that derived from expression of the RNAi construct. *ACTIN* primers spanning an intron were used to evaluate both the amount and quality of cDNA obtained. Since the *IDL* genes contain no introns, the *ACTIN* primers were also used to check for contamination with genomic DNA. *ACTIN* cDNA will not include the intron, and therefore produce slightly shorter fragments than the corresponding genomic PCR product. RT-PCR was run at a variety of cycles between 25 and 35. When cDNA from seedlings were run at 30 cycles, a clear band was visible in wildtype cDNA, whereas there was only a faint band from the RNAi sample (progeny of T<sub>2</sub> plant 10). A difference in band intensity could not be detected between the wildtype and RNAi sample (progeny of T<sub>2</sub> plant 12) at 27 cycles, and at fewer cycles there was no band from either sample. The results from the RT-PCR with *ACTIN* primers are somewhat uneven, thus it may be speculative to argue any quantitative differences in expression levels of *IDL4* based on these data. Further studies are necessary to be conclusive, perhaps with more quantitative methods like northern or real-time PCR. Researchers have also reported reduced protein levels from RNAi constructs, with no detectable difference in mRNA levels (Hannon and Rossi 2004), thus proteomic methods such as western blotting may be required to show a reduction in expression. No aberrant phenotypes were detected for the *IDL4* RNAi transformants.

Genomic DNA	wt buds	line 12 buds	wt seedl.	line 10 seedl.
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**Fig 3.13 Semi-quantitative RT-PCR**

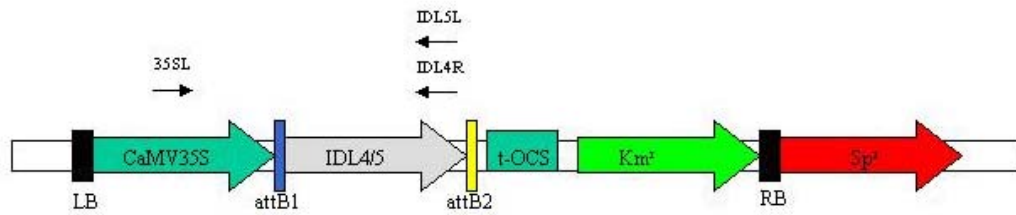
PCR run on cDNA from both seedlings and buds with various numbers of cycles. At 31 cycles (top row, all bands 387 bp), *IDL4* cDNA was detectable in all lines, however very faint in the seedling sample from RNAi line 10. At 28 cycles (middle row, all bands 387 bp), *IDL4* cDNA traces were found in the bud sample from both wild type and RNAi. The bottom row shows PCR products with *ACTIN* primers of similar amounts from the various cDNA samples (21 cycles, inverted photo. Genomic band 339 bp, RT-bands 253 bp).

### 3.4 Gain-of-function analysis

One way to elucidate the function of a given gene is to manipulate plants to increase the expression of it, and look for abnormal phenotypes. Cauliflower Mosaic Virus promoter 35S is a strong promoter that functions in all plants. The 35S promoter will direct expression of any gene cloned in front of it in most tissues of a transformed plant.

#### 3.4.1 The over-expression constructs

The over-expression constructs were made using the Gateway cloning system (see chapter 2.4.5). The coding *IDL4* and *IDL5* sequences were cloned down stream of the strong promoter CaMV 35S in the vector pK7WG2 (fig 3.14). PCR with gene-specific primers and DNA sequencing with the primer 35SL were used to screen for positive bacterial clones. *Arabidopsis* Col-0 plants were transformed using the *Agrobacterium* flower dip method (see paragraph 2.1.6). For each construct, 45 primary transformants were obtained. To examine whether the transformed plants showed increased transcription of the relevant genes, semi-quantitative RT-PCR was performed.

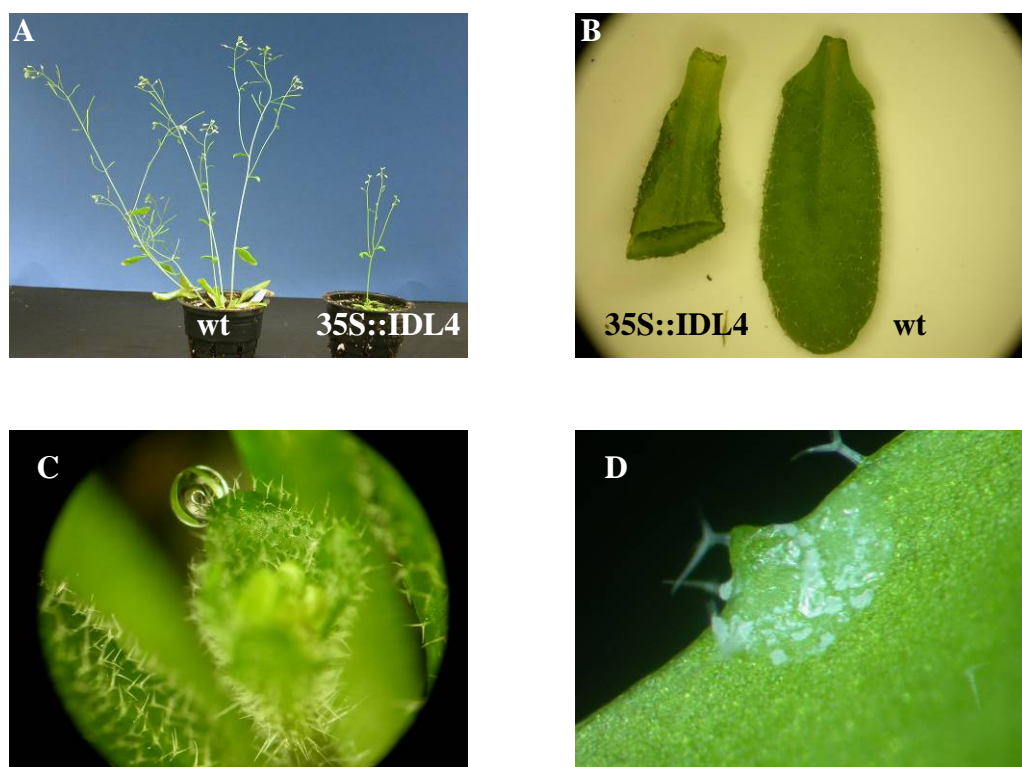


**Fig 3.14 The pK7WG2 over-expression constructs for IDL4 and IDL5**

The coding region of the genes are cloned down stream of the CaMV 35S promoter. The constructs transformed to plants also include a 35S terminator, a kanamycin resistance gene with promoter and terminator, Right and Left borders. The gene of interest is flanked by att sites. To aid selection in bacteria, the vector contains a Spectinomycin resistance gene.

#### 3.4.2.1 Investigating the phenotype of the *35S::IDL4* transformants

T1 plants were selected on MS-2 media with 50 µg/l kanamycin. 45  $Km^r$  plants from several independent transformants were transferred to soil and investigated for abnormal phenotypes. Mutant plants were smaller (Fig 3.16 A), and their leaves were curled and darker green compared with wildtype (Fig 3.16 B). The transformants flowered at the same time as the wildtype (data not shown). The *35S::IDL4* plants also showed increased guttation from the hydathodes, with large drops of sap appearing at the leaf margins (Fig 3.16 C). Deposits of a white crystalline substance often appeared at the tips of rosette and cauline leaves (Fig 3.16 D). Some of the mutants did not have fully developed siliques and showed reduced fertility (data not shown). The severity of the mutant phenotype varied from almost indistinguishable from wild type to plants with rosette diameter less than 1 cm and inflorescence length below 5 cm. The latter corresponds to at least 5-fold decrease in size compared to wild type.

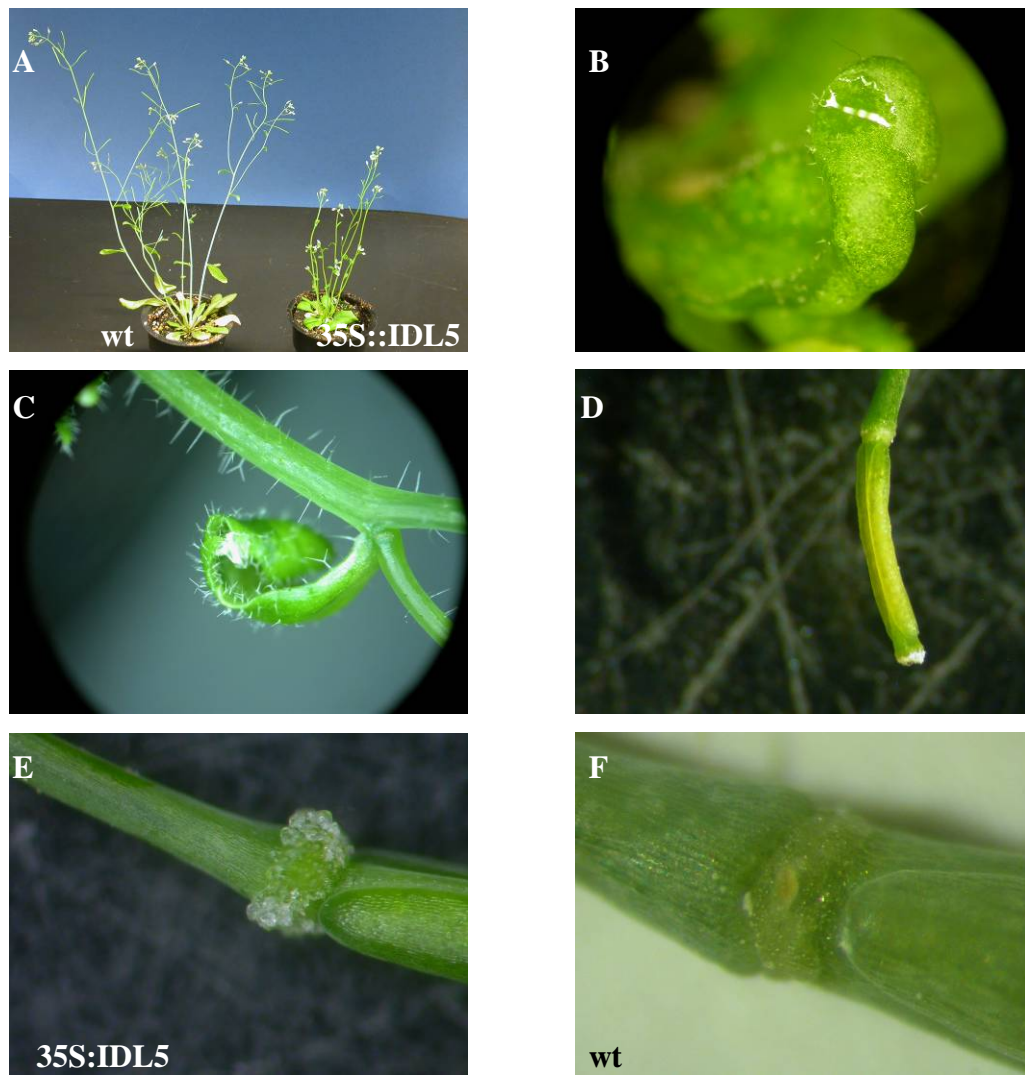


**Fig 3.16 35S::IDL4 mutant phenotypes**

Gain-of-function *IDL4* mutants were smaller (A) and had curled and darker green leaves (B) compared to wildtype. The transformants also showed increased guttation (C), and white, crystalline deposits often appeared on the leaf margins (D).

### 3.4.2.2 Investigating the phenotype of the 35S::IDL5 transformants

As for the 35S::IDL4 transformants, 45 Kmr plants were transferred to soil and investigated. The abnormal phenotype for the plants with increased expression of *IDL5* was very similar to the ones mentioned for 35S::IDL4. The 35S::IDL5 plants were smaller in size (Fig 3.17 A), had curled and darker green leaves (Fig 3.17 B), increased guttation (Fig 3.17 B) and sometimes white, salty patches on the leaf margins (Fig 3.17 C). Some of the mutants developed abnormal siliques (Fig 3.17 D), and had reduced fertility. Interestingly, some (3/45) of the T<sub>1</sub> plants with increased *IDL5* expression developed enlarged FAZ with immense cell proliferation and a white substance seemed to cover the cells (Fig 3.17 E vs F).



**Fig 3.17 35S::IDL5 mutant phenotypes**

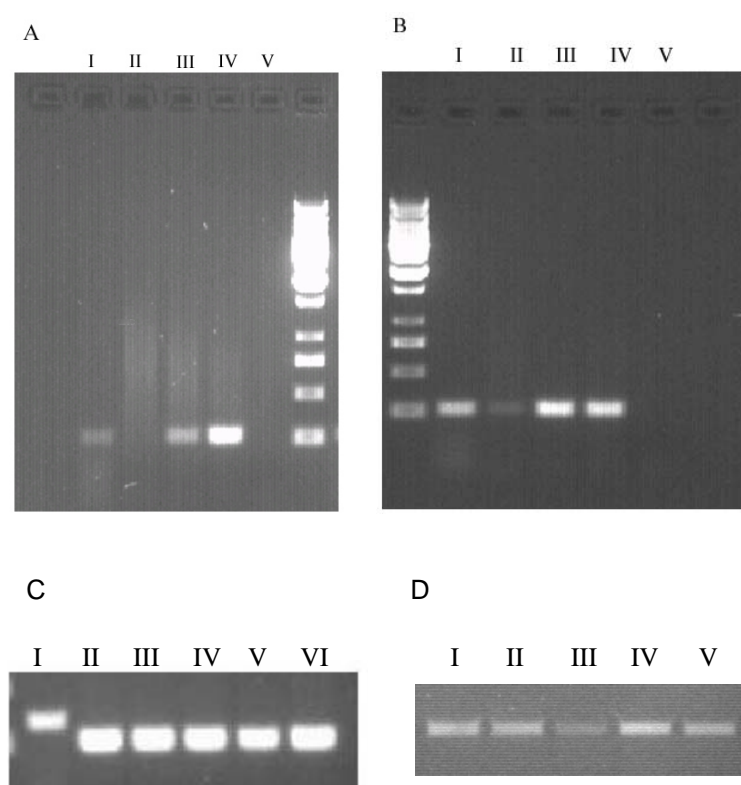
Gain-of-function *IDL5* mutants were smaller (A) and had curled and darker green leaves (B) compared to wildtype. The transformants also showed increased guttation (B), and white, crystalline deposits often appeared on the leaf margins (C). Some mutants developed abnormal siliques (D) and others appeared with enlarged floral AZ (E vs. F).

### 3.4.3 Semi-quantitative RT-PCR on 35S::IDL4 and 35S::IDL5

As previously described, semi-quantitative RT-PCR is a method to detect varying amounts of specific RNA present in tissue (chapter 3.3). Total RNA from rosette leaves of both primary transformants and wildtype plants was isolated, and first strand cDNA was synthesised. PCR with gene-specific primers was performed. The number of cycles in the PCR reactions was adjusted so that differences in amount of template could be detected, thus giving an estimate of the relative transcription levels of the given genes. *ACTIN* primers spanning an intron were



used to assay the quality and amount of first strand cDNA. *IDL4* cDNA was not detected in wildtype plants at 30 cycles, but clear bands were obtained from the *35S::IDL4* samples. The cDNA sample originating from the plant with the most severe phenotype, also showed the strongest *IDL4* band in the PCR. However, more experiments are needed to investigate the relationship between the severity of the phenotype and the amount of *IDL4* transcript. The wildtype first strand cDNA showed a faint band from PCR with the primers IDL5R and IDL5L. Both *35S::IDL5* cDNA samples produced clear *IDL5* bands at 30 cycles. No quantitative difference between the samples from plants with unlike degrees of phenotype was detected for *35S::IDL5*.



**Fig 3.18 RT-PCR on the *IDL4* and *IDL5* genes.**

**A:** gene specific primers IDL4L and IDL4R, bands 260 bp. I: Genomic DNA from wt Col-0. II: cDNA from wt Col-0. III and IV: cDNA from *35S::IDL4*. V: neg. control.

**B:** gene specific primers IDL5L and IDL5R, bands 258 bp. I: Genomic DNA from wt Col-0. II: cDNA from wt Col-0. III and IV: cDNA from *35S::IDL5*. V: neg. control.

**C:** Intron-spanning ACTIN primers with 30 cycles.

I: genomic wt (339 bp). II: cDNA from wt. III and IV: cDNA from *35S::IDL4*. V and VI: cDNA from *35S::IDL5*. (all cDNA bands 253 bp).

**D:** Actin primers with 21 cycles shows little variance between amounts of template cDNA

I: cDNA from wt. II and III: cDNA from *35S::IDL4*. IV and V: cDNA from *35S::IDL5*

### 3.5 Microarray analysis of the *idl5* mutant

It was shown in chapter 3.2 that the mutant with a transposon insertion in the *IDL5* gene (*idl5spm*) has a reduced level of *IDL5* mRNA in seedlings. However, there was no visible phenotype in the mutants in normal growth conditions. The *IDL* genes have extensive homology (Butenko et al. 2003), thus a lack of phenotype could be due to functional redundancy. Another possible explanation is that mutant plants change their gene expression to cope with the lack of *IDL5* transcript. Microarray is a powerful method to compare overall

transcription between biological samples. The technique was utilised to compare the transcriptome of the *idl5* mutant with that of wildtype, in search of molecular phenotypes.

### 3.5.1 Brief addressing of the technical procedure

cDNA was made from RNA samples of two-week old seedlings of *idl5* mutant and wildtype. The cDNA from the two samples were labelled with different fluorescent dye and hybridised to the same microarray slide. The slides contain more than 29 000 spots with 70-mer oligonucleotides, antisense to all annotated *Arabidopsis* genes, as well as various controls. Note that the *IDL* genes 2-5 are not represented on these slides, as they were not annotated at the time the slides were produced. After hybridisation, the microarray slides were scanned with a laser scanner, and computer software used to compare intensity levels of the different fluorescent dyes. The same software was used to discard poor spots, e.g. with unspecific or no hybridisation.

The datasets representing the hybridisation values were treated mathematically *in silico* to adjust for non-data sources of variance (normalization) and facilitate comparison between slides (scale fit). A t-test was used to identify genes that were differentially expressed in several slides. A cut-off value of 2-fold change in expression between mutant and wildtype was chosen.

### 3.5.2 Results from the microarray analysis

Wildtype Col-0 plants and *idl5* mutants were grown on plates as described in Material and Methods 2.1. Total RNA was isolated from 2-week old seedlings and reverse transcribed into fluorescence-labelled cDNA. This developmental stage was chosen, as both RT-PCR and promoter::*GUS* assay pointed towards *IDL5* activity. Two biological repeats were performed for each sample.

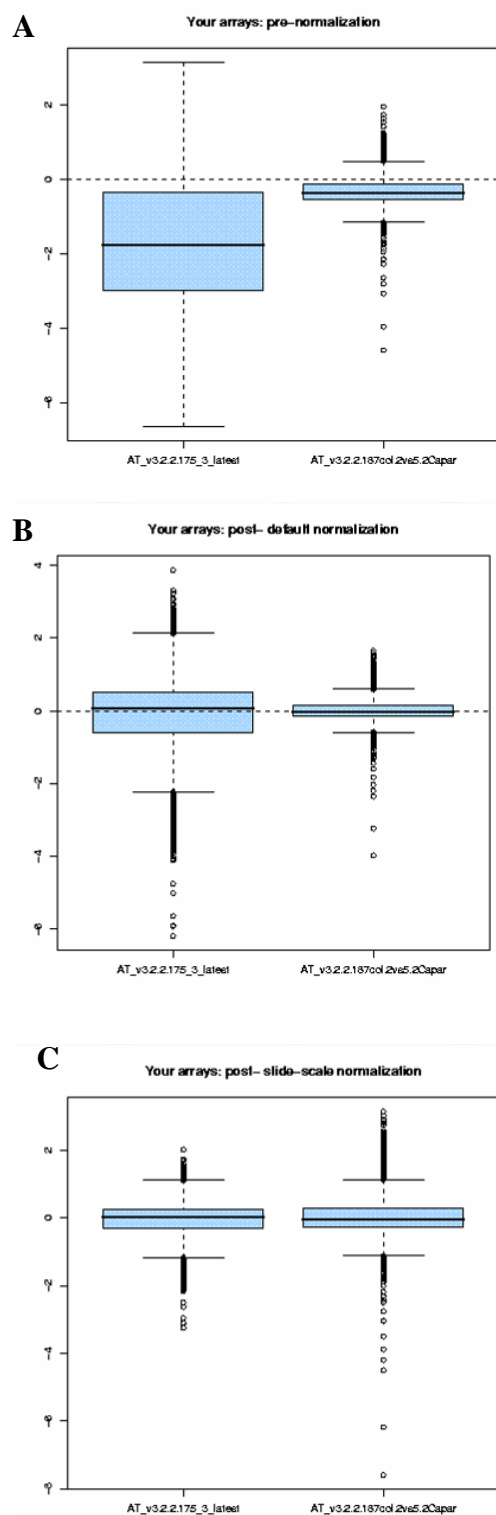
The two fluorescent dyes used in the experiment were Cy3 (green fluorescence) and Cy5 (red fluorescence). Slide AT\_v3.2.2.175 (hereafter called slide .175) was hybridised with Cy3-wildtype cDNA and Cy5-*idl5* cDNA. The dyes were swapped for slide AT\_v3.2.2.187 (hereafter called slide .187), i.e. this slide was hybridised with Cy5-wildtype and Cy3-*idl5* cDNA.



As described in Materials and Methods, the hybridised slides were washed and scanned with an Axon 4000B dual-laser scanner. The software GenePix 5.0 Pro (Axon Instruments) was used to analyse the images from the slides. Array elements with no detectable signal were flagged as not found by the software. Similarly, the software uses algorithms to flag elements spoiled by debris, scratches or with high local background as “bad” spots. Expression ratios were calculated as the mean of the ratios between Cy3 and Cy5 on a pixel-by-pixel basis.

DNMAD (<http://dnmad.bioinfo.cnio.es>) is a web-based software to normalise microarray expression data (Vaquerizas et al. 2004). Normalization is important to adjust for effects that could be explained in differences in technology, e.g. variance caused by difference in binding of the two dyes or background intensity. Figure 3.19 A and B show box plots of the ratio data from slide .175 and .178 before and after normalization, respectively. Note that after normalization, the median of the ratios has been adjusted to zero. From the box plots in B, it can clearly be seen that the two datasets are not in the same scale. Slide .175 seems to have a larger spread in the ratio values. This can be adjusted for by scale fitting (a separate function in DNMAD). The procedure does, however, produce additional noise in the data. Figure 3.19 C shows box plots of the ratios after scale fitting.

The normalised and scale-fitted ratio values from slide .187 were multiplied with  $-1$  to account for the dye-swap in the experiment. Datasets from both slides were imported into the software TMEV 3.1 (TIGR). This is open source software, which allows further statistical analysis of microarray data. A t-test was run in order to identify genes with expression significantly altered more than two-fold between wildtype and mutant in both slides. With a significance criterion of 0.05, this method identified 43 genes that were down regulated in the mutant, and one gene that was up regulated (table 3.3). The software GFINDER (Masseroli et al. 2004) was utilised to scan available databases, such as the gene Ontology database, of any known characteristics of the differentially expressed genes.



**Figure 3.19 Distribution of microarray data**

Boxplots representing the distribution of data from the two microarray slides. The values are  $\log_2$  ratios of the fluorescence intensity between the two dyes (red:green). A  $\log_2$  ratio of 0 represents no difference, a value of  $-1$  represents 2-fold greater intensity of green dye. **A**: ratios in raw data. **B**: ratios post normalisation. **C**: ratios post slide-scale normalisation

**Table 3.3 Genes with altered levels of cDNA in the *idl5* mutant**

Genes identified with t-test as being differentially expressed in the two replicated experiments. Biological process, Cellular component and Molecular function are as noted in the Gene Ontology database. Mean ratio –1 corresponds to a two-fold down-regulation in the *idl5* mutant, and mean ratio 1 corresponds to a two-fold up-regulation in the mutant. The genes marked in bold will be further discussed in chapter 4.

Gene ID	Gene Name/ family	Mean ratio	Biological process	Cellular component	Molecular function
<b>At5g49215</b>	<b>glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein</b>	-1.4764	carbohydrate metabolism, cell-matrix adhesion	endo-membrane system	polygalacturonase activity
<b>At1g70530</b>	<b>protein kinase family</b>	-1.1974	protein amino acid phosphorylation	endo-membrane system	kinase activity
<b>At4g28395</b>	<b>lipid transfer protein</b>	-2.0229	lipid transport, lipid binding	-	lipid binding
<b>At3g28080</b>	<b>nodulin MtN21 family protein</b>	-1.5276	sulfate transport	integral to membrane	sulfate porter activity
<b>At1g15150</b>	<b>MATE efflux family protein</b>	-1.0919	pathogenesis	integral to membrane	antiporter activity, drug transporter activity
<b>At3g45060</b>	<b>high-affinity nitrate transporter</b>	-1.0648	phosphate transport, inorganic anion transport	integral to membrane	nitrate transporter activity
At5g30520	-	-1.9251	-	-	-
At4g25400	basix helix-loop-helix (bHLH) family protein	-1.7470	-	formate dehydrogenase complex	DNA binding, transcription factor activity
At2g45550	cytochrome P450 family protein	-1.7026	electron transport	mitochondrion	mono-oxygenase activity
At5g11010	pre-mRNA cleavage complex-related	-1.5739	-	chloroplast	-
At4g21090	Ferredoxin, AtMFDX2	-1.5118	-	mitochondrion	electron carrier activity, iron ion binding
At1g69580	myb family transcription factor	-1.3540		nucleus	transcription factor activity
At5g07100	WRKY family transcription factor	-1.3294	regulation of transcription, DNA-dependent	mitochondrion	transcription factor activity
At4g12310	cytochrome P450, putative	-1.3187	electron transport	mitochondrion	monooxygenase activity

Gene ID	Gene Name/ family	Mean ratio	Biological process	Cellular component	Molecular function
At4g30050	-	-1.3149	-	-	-
At3g14540	terpene synthase/cyclase family	-1.3140	terpenoid biosynthesis	chloroplast	lyase activity, sugar binding
At4g36910	CBS domain-containing protein	-1.2896	-	chloroplast	-
At1g54210	autophagy 12a (APG12a)	-1.2875	autophagic vacuole formation, protein ubiquitination	cytoplasm	protein binding
At4g00220	lateral organ boundaries domain protein 30 (LBD30)	-1.2709	-	chloroplast	-
At3g11520	Cyclin, CYC2	-1.216	regulation of cell cycle	nucleus	cyclin-dependent protein kinase regulator activity
At1g73360	homeobox-leucine zipper family protein	-1.2021	DNA transposition	nucleus	DNA binding
At2g46225	-	-1.1578	-	-	-
At5g40270	metal-dependent phosphohydrolase HD domain-containing protein	-1.1522	-	-	catalytic activity
At5g03960	calmodulin-binding family protein	-1.1418	-	mitochondrion	calmodulin binding
At3g42556	Protein of unknown function (DUF321)	-1.1319	-	-	-
At5g13840	WD-40 repeat family protein AtCcs52B	-1.1029		heterotrimeric G-protein complex	signal transducer activity
At1g48500	-	-1.0913	-	-	-
At1g35830	VQ motif	-1.0864	-	-	-
At1g15930	40S ribosomal protein S12 (RPS12A)	-1.0788	protein biosynthesis	-	structural constituent of ribosome
At1g03350	BSD domain-containing protein	-1.0750	-	-	-
At3g26330	cytochrome P450 family protein	-1.0694	electron transport	-	mono-oxygenase activity
At2g40450	speckle-type POZ protein-related	-1.0694	-	-	protein binding
At4g09740	glycosyl hydrolase family 9 protein	-1.0693	carbohydrate metabolism	endo-membrane system	hydrolase activity, hydrolyzing O-glycosyl compounds

Gene ID	Gene Name/ family	Mean ratio	Biological process	Cellular component	Molecular function
At5g20910	zinc finger (C3HC4-type RING finger) family	-1.0619	regulation of transcription (DNA-dependent), protein ubiquitination	ubiquitin ligase complex	DNA binding, Protein binding, Zinc ion binding, ubiquitin-protein ligase
At4g31790	diphthine synthase	-1.0569	peptidyl-diphthamide biosynthesis from peptidyl-histidine	-	diphthine synthase activity, S-adenosylmethionine-dependent methyltransferase activity
At4g10400	F-box family protein	-1.0499	mRNA metabolism	-	RNA binding
At2g40380	prenylated rab acceptor (PRA1) family protein	-1.0436	-	chloroplast	-
At3g42610		-1.0328	-	-	-
At1g05930	Domain of unknown function (DUF313)	-1.0244	regulation of transcription, DNA-dependent	-	DNA binding
At1g57590	pectinacetyl esterase	-1.020	-	endo-membrane system	carboxylic ester hydrolase activity
At1g30950	F-box family protein (FBX1)	-1.013	ubiquitin-dependent protein catabolism	ubiquitin ligase complex	ubiquitin-protein ligase activity
At4g05095		-1.0095	-	-	-
At5g49650	Xylulose kinase	-1.0030	D-gluconate metabolism, L-arabinose catabolism, xylulose metabolism	-	ribulokinase activity, xylulokinase activity
At1g14500	ankyrin repeat family protein	-1.0022	-	-	protein binding
At2g33310	Auxin-responsive protein IAA13 (Indoleacetic acid-induced protein 13)	-1.0017	-	nucleus	transcription factor activity
<b>At4g26530</b>	<b>fructose-bisphosphate aldolase</b>	1.1890	glycolysis	-	fructose-bisphosphate aldolase activity



## 4 DISCUSSION

The *IDA-LIKE* (*IDL*) genes were discovered based on their similarities to IDA, a putative peptide ligand shown to be involved in floral abscission in *Arabidopsis* (Butenko *et al.* 2003). Several plant species have transcripts encoding IDL proteins, and *Arabidopsis* contains at least 5 *IDL* genes. The expression pattern of the genes *IDL1-5* had been crudely investigated by RT-PCR. In this thesis, the *Arabidopsis* genes *IDL4* and *IDL5* were further investigated.

The expression pattern of *IDL4* and *IDL5* was examined more closely by histochemical assay of transgenic plants harbouring the expected promoter region fused to a reporter gene, *GUS*. Studying loss-of-function mutants is an invaluable tool in functional genetics. The *ida* insertion mutant shows a distinct phenotype in that floral organs failed to be shed. We examined plants with an immobilised transposable element situated within the *IDL5* cds and no detectable *IDL5* transcript compared to wt. The *idl5* mutant does not produce a striking phenotype. However, it was further studied with microarray transcriptional profiling. Since there was no insertion line available for *IDL4*, RNAi transgenic plants were constructed to induce specific gene silencing. Gain of function mutants for both *IDL4* and *IDL5* were constructed by fusing the genes downstream of the strong, constitutive 35S promoter.

### 4.1 The expression pattern of *IDL4*

The expression pattern of the gene *IDL4* was investigated with a histochemical GUS-assay as described in chapter 3.1. GUS activity was seen in a variety of tissues and organs during the developmental course of the plant (chapter 3.1.1).

#### 4.1.1 The *IDL4::GUS* expression predominated in young seedlings

*IDL4::GUS* expression was absent from seedlings until approximately 24 hours after germination, when it appeared in the most distal parts of vascular tissue in the cotyledons. 48-72 hours post germination expression was clearly detected in vascular tissue of leaves and roots, as well as in guard cells and hydathodes. The *IDL4* promoter activity seemed to decrease in roots and rosette leaves at later stages in development. In two-week old plants grown on plates, GUS activity was vividly present in vascular strands of rosette leaves and roots. However, in older rosettes (approximately four-week old) expression was restricted seemingly sporadic to hydathodes. Principal growth stage 1 (leaf development) in *Arabidopsis* is defined as the period between fully opened cotyledons, through rosette leaf development

until no new rosette leaves are formed (Boyes *et al.* 2001). The most prevalent expression pattern of *IDL4::GUS* falls within this growth stage, and could indicate that the *IDL4* is involved in forming new leaves and roots, rather than the growth and continuing function of these organs.

#### 4.1.2 *IDL4* expression was observed in stomata and hydathodes of young leaves

*IDL4::GUS* expression was seen in stomatal guard cells of developing seedling leaves, but not in either fully developed leaves or the inflorescence. Guard cells are kidney-shaped pairs that form the stomata, pores which facilitates gas exchange between the plant and its environment (Bergmann *et al.* 2004). Stomata are formed by symmetric division of a guard mother cell, which in turn was formed by asymmetric division of a precursor meristemoid cell (Nadeau and Sack 2002). A consistent feature of stomatal patterning is that the pores are separated by at least one cell (Sachs 1991). Several genes are known to control the development and patterning of stomata (Nadeau and Sack 2002), however a detailed description of this process would be beyond the scope of this discussion. One interesting pattern of the *IDL4::GUS* staining is that it in some cases was visible also in immediately adjacent cells that could resemble the sister cell of the guard mother cell (Zhao and Sack 1999). If *IDL4* is expressed in stomatal complexes during differentiation, *IDL4* could be involved in the formation of stomata, rather than the continuing function of these structures. Hydathodes are specialised stomata at the leaf margins where guttation occur, but the structures probably do not follow the spacing criteria mentioned for stomatal pores (Nadeau and Sack 2002). In the *IDL4::GUS*-assay, hydathode staining was observed consistently in young, developing seedlings, but only sporadically in fully-grown rosette leaves. The feature that *GUS* expression appeared in guard cells and stomata predominantly during leaf development could indicate that *IDL4* is involved in the formation or patterning of these structures, rather than in their day-to-day function.

Stomatal development is associated with the specific breakdown of the wall joining the two guard cells together (Zhao and Sack 1999), and it is likely that cell separation processes occur during the formation of hydathodal pores as well (Roberts *et al.* 2002). Another attribute to formation of the pores is the likely up regulation of genes in pathogen defence-responses, in order to protect exposed parts of the plant from invading microbes. *IDL4* could be involved in such processes, which would also partly explain the *GUS* activity observed in hydathodes of fully-grown leaves.



#### 4.1.3 *IDL4* expression was observed in the funicles and seed abscission zones

*IDL4::GUS* expression appeared in the funicles and SAZ, and could imply that *IDL4* is somehow involved in seed dispersal. The transcription factor SEEDSTICK (*STK*) is required for normal development of the funicle and SAZ, and is expressed in these structures (Pinyopich *et al.* 2003). The polygalacturonase (PG) *SAC70* (a.k.a. *ESJ2 A*) (Jenkins *et al.* 1999) is another *Arabidopsis* gene that showed staining in the SAZ in a GUS assay (the authors investigated the *Arabidopsis* promoter in *B. napus* transformants), thereby showing some overlap with the results for *IDL4*. The exact function of *SAC70* is not known, but Jenkins and colleagues hypothesise that it is involved in the break-down of cell walls in seed abscission and pod dehiscence, and that these phenomena may be brought about by a common signal (Jenkins *et al.* 1999). Interestingly, a *SAC70* homologue *ADPG1*, has been shown to be expressed in the adaxial part of the branching point between stem and pedicel (Sander *et al.* 2001). This was also observed for *IDL4*. *Arabidopsis* does not have naturally occurring pedicel abscission. However, several close relatives do have functioning pedicel AZ, and *Arabidopsis* may retain evolutionary relics of a pedicel abscission process (Cho and Cosgrove 2000).

#### 4.1.4 *IDL4* expression in roots and carpels

The *GUS* reporter gene was activated in vascular parts of both primary and lateral roots. We did not establish in which of the vascular cell types the GUS activity was present. One interesting pattern, however, was that while expression in primary roots was limited to the differentiated vascular parts, lateral roots showed expression in the meristematic root tip as well (fig 3.3). A similar expression pattern has been noted for *IDL2* (Tandstad 2005). Our results could tentatively indicate that *IDL4* serves different functions in primary and lateral roots. However more detailed studies of the *IDL4* expression pattern need to be undertaken to validate this hypothesis. For instance, the *GUS* expression in vascular tissue could be further investigated by longitudinal thin-sectioning stained roots. The staining in lateral root tips could be studied in more detail by co-staining with Lugol solution, thereby visualizing specific columella cell layers.

During the early flower stages, *IDL4::GUS* expression was seen in the carpel wall. Some time before floral organ abscission expression in the ovaries ceded, and through subsequent stages of flower and fruit development, GUS was observed at the base of siliques and the style as seen in figure 3.4. It is very interesting to note that the *STK* gene involved in funicle and SAZ

development is also expressed in young ovule primordia within developing carpels. It is possible that *IDL4* and *STK* function in a common developmental pathway, and it could be worthy considering further studies of *IDL4* in the *stk* background.

#### **4.2 The expression pattern of *IDL5***

Analysis of the *IDL5::GUS* fusion transformants showed activity in developing rosette leaves. GUS staining was not evident until some time after the cotyledons were fully opened (fig 3.5). Expression of the reporter gene was stronger in younger, not fully expanded leaves, where it could be seen across the surface of the leaf. The activity decreased in elder leaves, and became concentrated in vascular tissue and hydathodes. In fully developed rosette leaves, the pattern seemed to be restricted to sporadic expression in hydathodes. No GUS activity was shown in roots. Various adult tissues were tested in the GUS assay, but no expression was evident there.

The promoter activity in developing leaves could imply that *IDL5* is somehow involved in the processes that lead to leaf maturity. The expression pattern from ubiquitous activity in emerging leaves toward vascular parts of elder leaves could indicate that the gene is engaged in vascular patterning and/or differentiation of vascular tissue.

#### **4.3 Pathogenesis and injury**

*IDL4* and *IDL5* are expressed in parts of the plant where cell separation processes occur, however cells in these tissues also share the characteristic that they are unprotected by mechanical barriers and hence susceptible to microbial attack (Hong *et al.* 2000; Lorrain *et al.* 2004). Several pathogenesis-related (PR) genes have been shown to be expressed in AZs and other sites where the protective cell wall is modified or lost (Hong *et al.* 2000), or where the interior of the plant is in contact with the environment through pores such as stomata and hydathodes (Chen *et al.* 2003; Kobae *et al.* 2006). We investigated if the promoters of *IDL4* and *IDL5* could be further activated upon pathogen infection.

##### **4.3.1 GUS assay following *P. syringae* infection**

*P. syringae* enters the host tissues, usually leaves, through wounds or natural openings such as stomata and multiplies in the intercellular space if the plant is susceptible. The *IDL4::GUS* and *IDL5::GUS* transformants showed normal disease symptoms upon pressure infiltration with bacterial suspension. However, the results from GUS assay of infected leaves were

somewhat ambiguous. Some, but not all leaves of *IDL4::GUS* showed increased *GUS* expression at the site of infiltration. Plants were also treated with a sterile buffer solution, and this did also promote increased *IDL4::GUS* expression in some cases. From this, one can conclude that it is unlikely that the *P. syringae* itself is the causing agent for increased expression. One cannot entirely rule out that the suspension and control buffer utilised were contaminated with a bacterium other than *P. syringae*, and thus mounted a separate defence response in the plants. Some bacteria have intrinsic *GUS* activity (Jefferson 1989), and contamination with such microbes could also explain the observed pattern. It is also possible that the syringe infiltration was done forcefully enough in some cases to cause wounding, and that the *IDL4* promoter became activated in a response to this injury.

#### 4.3.2 GUS assay following *B. cinerea* infection

Fungal hyphae of the necrotrophic pathogen *B. cinerea* enters the plant through wounds or naturally exposed cells (Cristescu *et al.* 2002), and the microbe secretes toxins to actively kill plants cells. In our experiments, *B. cinerea* infection did not cause elevated levels of *GUS* expression in either *IDL4::GUS* or *IDL5::GUS* transgenic plants. Thus it is not likely that either *IDL4* or *IDL5* is involved in defence responses against such pathogens.

#### 4.3.3 GUS assay following wounding

Partly based on the inconsistent data from the *P. syringae* assay, we examined whether *GUS* activity could be induced in leaves by merely wounding the tissue. Plants alter their gene expression upon wounding, both to regenerate continuous vascular tissue (Berleth *et al.* 2000) and as a defence against microbes on the exposed surfaces (Cheong *et al.* 2002). Initial results indicated that *IDL4::GUS* activity could indeed be induced by wounding (fig 3.6). When cut across the midrib, *GUS* expression was visible solely in the vascular tissue surrounding the site of injury. However, when the experiment was repeated, we did not obtain the same pattern. Thus, based on our findings it is unlikely that *IDL4* has a role in a general wounding response. The staining pattern initially observed could be explained by contamination with *GUS*-expressing bacteria.

#### **4.4 Investigating loss-of-function mutants for *IDL4* and *IDL5***

##### **4.4.1 Investigating possible insertion mutants and RNAi silencing transformants for *IDL4***

We isolated a SALK insertion line with a T-DNA inserted in the vicinity of *IDL4*, however sequencing of the genomic region flanking the T-DNA left border located the insertion to 83 base pairs downstream of the *IDL4* cds. It is unlikely that this would produce a loss-of-function mutation of the gene, and it could explain the lack of a detectable phenotype in homozygous SALK lines.

Since we could not attain a positive insertion line for *IDL4*, an RNAi knockdown line was created with the vector pHELLSGATE8 (chapter 3.3). No mutant phenotype was observed for the transformants, and results from semi-quantitative RT-PCR was not sufficiently clear to conclude how successful the silencing of the *IDL4* gene had been.

The lack of effective gene silencing in the RNAi transformants could be due to a variety of phenomena. The silencing efficiency has been shown to vary greatly between individual transformants (Wang *et al.* 2005). Variability in how efficiently gene silencing is inherited to subsequent generations has also been noted (Wang *et al.* 2005). Some authors have proposed a negative relationship between copy-numbers and degree of silencing (Kerschen *et al.* 2004), but there is still some controversy about these conclusions (Wang *et al.* 2005). It is also possible that the 35S promoter driving the expression of hair-pin RNA is not equally effective in all tissues (Chuang and Meyerowitz 2000), thus gene silencing may not occur in the appropriate cells. RNAi seldom cause complete elimination of gene expression (Wesley *et al.* 2001), and some phenotypes could be less sensitive to gene activity levels (Chuang and Meyerowitz 2000). Lastly, RNAi acts by decreasing the half-life of RNA, thus transcripts that are rapidly produced and degraded are likely to be less effected (Fire 1999). If the assumption that the *IDL* genes encode signal peptides that act in carefully controlled cellular processes is correct, they are likely to have a short half-life and therefore not effectively silenced post-transcriptionally.

##### **4.4.2 Investigating an *idl5* insertion mutant**

A SLAT line with the immobilised dSpm element to be situated within the *IDL5* cds was acquired. Homozygous plants were obtained, and RT-PCR results were consistent with the lack of *IDL5* transcript in seedlings (chapter 3.2.3). No aberrant phenotype was detected when the *idl5* plants were grown in normal conditions. It is likely that plants, being sessile

organisms, have evolved numerous traits that make them able to cope with severely changing environments. However, a careful and systematic analysis of many interacting biotic and abiotic factors would be beyond the scope of this project - even outside the possibilities of most research groups (Bouché and Bouchez 2001).

A second possibility that could explain the lack of phenotype in *idl5* is functional redundancy. *IDL5* belongs to a relatively closely related group of genes (chapter 1.8.2), and some of the other *IDL* expression patterns may overlap with that of *IDL5* both spatially and temporally. Results from GUS-assays imply that *IDL2*, *IDL3* and *IDL4* are all expressed in vascular strands of cotyledons, and to a varying degree also in emerging rosette leaves ((Tandstad 2005) and chapter 3.1.2 in this thesis). Overlapping expression of genes encoding similar proteins could cause a loss-of-function mutant in several of the *IDL* genes to be required in order to obtain informative phenotypes (Finkelstein *et al.* 2005).

#### 4.4.3 Microarray analysis of the *idl5* mutant

Microarray was used to compare the expression profiles of the *idl5* mutant with wild type (chapter 3.5). From the set of experiments, we obtained a list of genes containing 45 genes significantly down regulated and a single gene that was up regulated in the mutant. Databases were searched for known characteristics of the differentially expressed genes (table 3.3). No palpable pattern of function was observed from the collection, with genes involved in biological processes as diverse as electron transport, regulation of cell cycle, pathogenesis, ribosomal constituents, protein ubiquitination, transport of ions and carbohydrate metabolism. However, some of the genes are worth to mention based on other results obtained thus far. *IDA* is involved in a cell separation process that involves polygalacturonases (PG) for degrading the cell wall between adjacent cells. One PG gene, *At5g49215*, with unknown function seemed to have a lower expression in the *idl5* mutant. It could be speculated that this PG functions in some kind of cell separation process downstream of an *IDL5*-evoked signal. On the other hand, among the candidates for down regulated genes in the *idl5* mutant there were several transporters such as for lipids, sulphate and nitrate. Transporters has been linked to hydathode function (Pilot *et al.* 2004) – structures where *IDL5::GUS* activity was evident.

There are several considerations one needs to contemplate with this approach to obtain clues about gene function. Firstly, it could be argued that repeating the procedure merely once is not sufficient to provide a reliable basis for functional analysis. Microarray data are

characterised by a vast amount of variables (genes) and only a few observations of each variable. Although statistical tools are used to assess this problem, there is still a chance to accumulate false positives. It was also evident that there was some variance between the distributions of ratios from the two hybridisation experiments (fig 3.19). A second normalisation step (slide-scale normalisation) was needed to make the data comparable, and this produced additional noise in the data.

*IDL5* mRNA was detectable by RT-PCR on seedling tissue, but the promoter::GUS assay suggested that the gene is not strongly expressed in all parts of the seedling. There is a possibility that molecular phenotypes in minor parts of the plant is masked by the expression in the rest of the plant.

Microarray is a powerful method for the initial functional characterisation of unknown genes, however secondary information is required to validate any findings. Techniques like RNA gel blots (northern) and quantitative PCR with real-time instruments (Richmond and Somerville 2000) could be undertaken. Unfortunately we did not have the time available to perform secondary experiments on *idl5* expression.

#### **4.5 Over expressing the *IDL4* and *IDL5* genes**

Over expression of the two genes led to almost indistinguishable phenotypes (chapter 3.4), however the severity ranged from severe to wild type-like between individuals from both constructs. Plants were generally smaller, and had curled leaves that appeared water-soaked. They also showed increased guttation and white, crystalline patches often appeared on leaf margins. Some transformants developed abnormal siliques and showed reduced fertility. A minority of the individual plants over expressing *IDL5* developed enlarged FAZs and cells there were covered in a white substance. Results from semi-quantitative RT-PCR were consistent with increased transcription of the respective gene in plants transformed with either the *35S::IDL4* or the *35S::IDL5* construct.

The abnormally shaped leaves could be further indication that both genes in question may be involved in leaf development, as was speculated based on the GUS assay. The over expressing plants seemed to have normal vascular patterning, however. No differences in stomatal patterning or seed abscission was observed, however these traits would need more careful analysis to be conclusive.

A more likely explanation of most aspects of the phenotypes is increased activity in hydathodes. Increased differentiation of hydathode structures and/ or increased activity could account for the increased guttation. This could also explain the salty patches on leaf margins, which may be due to evaporation of water in the excreted sap. It could also be speculated that the water-soaked appearance of the leaves is due to cells performing excretion elsewhere in the leaf. It is interesting to note that both *35S::IDL4* and *35S::IDL5* closely resemble plants over expressing *GLUTAMINE DUMPER1 (GDU1)*, in being smaller in size, having curled and darker green leaves, increased guttation and salty patches on leaf margins (Pinyopich *et al.* 2003). *GDU1* has been shown to function as an amino acid transporter membrane protein, and the increased guttation and salty deposits were explained by elevated export of glutamine.

The over expression phenotypes of *IDL4* and *IDL5* are very similar, even though the two genes seemed to be expressed in rather different tissues. The 35S promoter leads to constitutive expression in most tissues, and could cause the predicted ligands to react with each other's or a common receptor. Functional redundancy is common in higher plants, and it has been shown that several members of the CLAVATA3-related (CLE) family can rescue the *CLV3* loss-of-function phenotype (Ni and Clark 2006). Such redundancy could also explain the abnormal FAZ seen in some of the plants over expressing *IDL5*. *IDA* is a related protein involved in floral abscission, and all *35S::IDA* transformants bear a similar trait (Stenvik 2006). Similar FAZ was seen in plants over expressing *IDL1*, 2 and 3 (Tandstad 2005).

The *IDL* genes are thought to contain a signal peptide targeting the proteins to the intercellular space (chapter 1.8). At this stage, it cannot be ruled out that the observed phenotypes are caused by a general stress response not directly related to the genes' normal function, e.g. from large quantities of secreted protein in the apoplast. Plants should also be transformed with an empty pK7WG2 vector to rule out that it is the vector itself that causes the phenotypes. It has been proposed that over expression of proteins being transported to the apoplast can be recovered efficiently in the guttation sap of tobacco (Komarnytsky *et al.* 2000). It would be interesting to analyse the guttation fluid in *35S::IDL4* and *35S::IDL5* for the presence of *IDL* proteins. Guttation fluid could be harvested without devastating the plant and analysed with methods such as western blots.

#### 4.6 Conclusive remarks and future aspects

Tools in reverse genetics have been utilised to examine the expression pattern and function of the *Arabidopsis* genes *IDL4* and *IDL5*. Promoter activity analysis has shown gene function in a wide range of tissues to be probable. Noticeably the *IDL4* promoter was activated in tissues throughout the life span of the plant, whereas *IDL5* activity was shown exclusively in young parts. One could tentatively suggest that the *IDL* genes show activity at sites where cell separation processes occur, however expression was not restricted to such regions and this hypothesis would require further assessment.

*IDL4* and *IDL5* may also be involved in the formation and/ or function of hydathodes. Both genes' promoter showed activity in these pores, and the gain-of-function mutants displayed increased guttation.

*IDL4* and *IDL5* are members of a group of at least 6 very similar proteins in *Arabidopsis*. The lack of apparent phenotypes in loss-of-function mutants could suggest functional redundancy within this group. This could also explain the highly similar phenotypes observed upon over expressing *IDL4* and *IDL5*.

The *IDL* genes are believed to encode secreted proteins that act as ligands in cell-to-cell communication. At present, however, no direct evidence exists that *IDL4* and *IDL5* are localised in the intercellular space. If the *IDL* genes do indeed encode peptide ligands, one should next attempt to identify their receptors. The *Arabidopsis* genome is thought to encode more than 600 receptor-like kinases, the vast majority of which ligands are still unknown.

In the event of further pursuit of *IDL4* and *IDL5* function, the GUS-assay should be more carefully assessed. The timing of expression in seedlings for both constructs and floral organs of *IDL4::GUS* could be elucidated in detail. Longitudinal sectioning of leaves and roots expressing GUS could provide more precise information of which cell types show promoter activity.

The RNAi silencing of *IDL4* expression is, at best, varying. It would therefore be advantageous to work with complete knockout lines. The small size of the gene's coding region decreases the probability of finding random insertion mutants. TILLING (Targeting Induced Local Lesions IN Genomes) is a relatively new strategy to induce and identify point



mutations, small deletions or insertions in specific regions or genes (McCallum *et al.* 2000). Perhaps it would be possible to obtain loss-of-function *idl4* mutants with this method. Because of the possibility of functional redundancy between the IDL proteins, it may be necessary to construct double or manifold mutants to elucidate the functions of the genes. Clues of redundant functions could come from a domain-swap screen. It has been shown that related motifs from several members of the CLE family can more or less rescue the CLV3 phenotype (Ni and Clark 2006). For the moment only *ida* has a well-characterised phenotype, but it would be interesting to see if any *IDL* genes driven by *IDA* regulatory sequences could make *ida* plants abscise their floral organs.

The *idl5* insertion mutant could be further investigated. Based on both GUS results and the gain-of-function phenotype, it would be interesting to look closer at hydathode formation. Transgenic *Arabidopsis* plants with a GUS-based marker for hydathodes denoted as #1-35-38 exist (Tsukaya and Uchimiya 1997), and these plants could be crossed into the *idl5* background to aid the visualisation of hydathodes in the mutant.

The #1-35-38 hydathodal marker could also be useful in further analyses of the *35S::IDL4* and *35S::IDL5*. It would be of particular interest to investigate whether increased guttation is linked to enlarged hydathodes or cells differentiating to hydathodal structures where they would not in wildtype plants. It is also possible that the over expression causes the gene product in question to be excreted, or that it activates another transport mechanism. Chemical analyses of the guttation sap may help cast light on which processes could be involved (Pilot *et al.* 2004). The salty patches on leaf margins could be examined by flame spectrometry for inorganic ion content. Mass spectrometry can further assess the possible atomic size of the contents in the sap, and protein gel analysis could evaluate if the sap contains proteins. *IDL4::GUS* expression occurred in stomata, and it would be interesting to closely inspect stomatal shape and patterning in *35S::IDL4*. This could be done with electron microscopy (EM) – Scanning EM for inspection of surface structure and stomatal patterning, and Transmission EM for features such as guard cell wall shape.

*IDL4* and *IDL5* have been assigned the standard ORF names At3g18715 and At1g76952, respectively, but are still pending to be implemented in the genomic databases as such. When this is done, it is likely that more data will accumulate, e.g. from microarray expression

profiling of various developmental stages or stress conditions. Thus more educated guesses regarding further functional studies of the genes can be made in the future.

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Primer name	Primer sequence	Tm (°C)	Primer usage
AtIDL5R	GGTGTTCATGGAGGATTTG	50.9	Gene- spesific, IDL5
AtIDL5L	TCATGGACATCTATGGGAGTTAG	50.2	Gene- spesific, IDL5
Spm1	CTTATTTTCAGTAAGAGTGTGGGGTTTTGG	59.6	IDL5 insertion
IDL5LP	ATGTTTTGTAGAAACCCAAGC	49.8	IDL5 insertion
IDL5RP	ATGGTACGCATTTGTTGGTG	50.8	IDL5 insertion
AtIDL4R	CAACAAGGCTTGAATACCAATG	51.7	Gene- specific, IDL4
AtIDL4L	CGTCCACATTATTGGAGAAGAAG	52.4	Gene- specific, IDL4
35SL	CAACCACGTCTTCAAAGCAA	51.2	Sequencing
M13 forward	GTAAAACGACGGCCAG	43.6	Sequencing
M13 reverse	CAGGAAACAGCTATGAC	36.2	Sequencing
smGFP <sub>r</sub>	CCGTATGTTGCATCACCTTC	50.3	Sequencing the pKEGAW vectors
HU	AAAGGGGGATGTGCTGCAAGGCG	66.6	PCR- testing pHellsgate8
HR	GCTTCCGGCTCGTATGTTGTGTG	60.4	PCR- testing pHellsgate8
Lbb1	GCGTGGACCGCTTGCTGCAACT	65.1	Genotyping SALK- lines
Lba1	TGGTTCACGTAGTGGGCCATCG	61.4	Genotyping SALK- lines
IDL4RP	TCGTGGTAGTATAACAACCTCTGGG	53.5	IDL4 SALK line genotyping
IDL4LP	TCCCACTTCCTCAAACTGAAACA	56.6	IDL4 SALK line genotyping
3-UTR_IDL4	TGAGTGCATGCATTAGATAT	42.9	RT-PCR, IDL4RNAi
5-UTR_IDL4	AAATCCCCTTCCTCAAAAC	47.5	RT-PCR, IDL4RNAi
3-UTR_IDL5	TCTGTAGATGAAGATCATGGACAT	51.4	RT-PCR, IDL5RNAi
5-UTR_IDL5	CTGTTTTCTGAAAGCAAGCT	46.7	RT-PCR, IDL5RNAi
IDL4attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGC ATGTATCCTACACGTCCACATTATTGG	56.4*	Gateway cloning IDL4
IDL4attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTAA GGAGACAACAAGGCTTGAATACCAATG	60.7*	Gateway cloning IDL4 (no stop codon)
IDL5attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGC ATGGGCAATAAACGCATAAAAGC	56.3*	Gateway cloning IDL5
IDL5attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTAT GGACATCTATGGGAGTTAGTACTCTG	54.1*	Gateway cloning IDL5 (no stop codon)
IDL4attB2 stop	GGGGACCACTTTGTACAAGAAAGCTGGTCAA GGAGACAACAAGGCTTGAATACCAATG	61.7*	Gateway cloning IDL4
IDL5attB2 stop	GGGGACCACTTTGTACAAGAAAGCTGGTCAT GGACATCTATGGGAGTTAGTACTCTG	54.6*	Gateway cloning IDL5
Ubiquitin left	CACACTCCACTTGGTCTTGCGT	56.5	RT-PCR
Ubiquitin right	TGGTCTTTCCGGTGAGAGTCTTCA	58.8	RT-PCR
Actin2int2_antisense	CCGCAAGATCAAGACGAAGGATAGC	54.2	RT-PCR
Actin2int2_sense	CCCTGAGGAGCACCCAGTTCTACTC	57.5	RT-PCR
*Calculated for the annealing part of the primer			



## ABBREVIATIONS

35S	<i>Cauliflower mosaic virus</i> 35S promoter
aa	amino acid
AA-dUTP	aminoallyl dUTP
<i>Agrobacterium</i>	<i>Agrobacterium tumefaciens</i>
<i>Arabidopsis</i>	<i>Arabidopsis thaliana</i>
avr	avirulence
AZ	Abscission zone
<i>B. cinerea</i>	<i>Botrytis cinerea</i>
<i>B. napus</i>	<i>Brassica napus</i>
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	Bovine serum albumin
cDNA	complementary DNA
cds	coding sequence
<i>CLE</i>	<i>CLAVATA3/ESR-RELATED</i>
<i>CLV</i>	<i>CLAVATA</i>
Col	Columbia
Cy3	Cyanine3
Cy5	Cyanine5
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsRNA	double stranded RNA
dUTP	deoxyuridine triphosphate
<i>DVL</i>	<i>DEVIL</i>
<i>E. coli</i>	<i>Escherichia coli</i>
EtBr	ethidium bromide
EM	electron microscopy
EtOH	ethanol
ETR1	"ethylene insensitive"
FAZ	Floral abscission zone
<i>GDU1</i>	<i>GLUTAMINE-DUMPER1</i>
GUS	β-glucuronidase
hpRNA	hairpin RNA
<i>IDA</i>	<i>INFLORESCENCE DEFICIENT IN ABSCISSION</i>
<i>IDL</i>	<i>IDA-LIKE</i>
JA	Jasmonic acid
kb	kilo base
Km	Kanamycin
Kmr	Kanamycin resistant
Kms	Kanamycin sensitive
LB	T-DNA left border
LRR	Leucin-rich repeat
mQ	milli-Q
mRNA	messenger RNA
nos	nopaline synthetase

<i>nptII</i>	<i>Neomycin phosphotransferase gene</i>
OD	optical density
ON	over night
ORF	Open reading frame
<i>P. syringae</i>	<i>Pseudomonas syringae</i>
PCR	Polymerase Chain Reaction
PG	Polygalacturonase
pI	Isoelectric point
PN	Plant nutrient
PSK	Phytosulfokine
pTi	tumor-inducing plasmid
pv	Pathovar
RB	T-DNA right border
R-genes	Resistance genes
RISC	RNA-induced silencing complex
RLCK	Receptor-like cytoplasmic kinase
RLK	Receptor-like kinase
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
RT-PCR	Reverse transcriptase PCR
SA	Salicylic acid
SAZ	Seed abscission zone
SCR	S-LOCUS CYSTEINE-RICH
SDS	sodium dodecyl sulfate
ser	serine
siRNA	small interfering RNA
Spm	Suppressor-mutator
SSC	Saline-sodium citrate
<i>STK</i>	<i>SEEDSTICK</i>
T1	first transformant generation
T2	second transformant generation
T3	third transformant generation
T-DNA	transfer DNA
thr	Threonine
Tm	melting temperature
tnp	transposase
UCT	University of Cape Town
UiO	University of Oslo
UTR	untranslated region
vir	virulence
wt	wildtype
X-gluc	5-bromo-4-chloro-3-indolyl $\beta$ -D-glucuronide